EFFECTS OF EXCESSIVE NATURAL MULCH ON DEVELOPMENT, YIELD, AND STRUCTURE OF NATIVE GRASSLAND[1,2]

J. E. WEAVER AND N. W. ROWLAND

Introduction

Prairie vegetation is so universally mowed, grazed, or burned that little information has been recorded on its behavior under complete protection. Climax grassland, when grazed lightly or even moderately, may retain essentially its natural composition over extremely long periods (**16, 18**). But aside from observation there is little information upon the unusual and rare phenomenon of complete protection of grassland from disturbance over a considerable period of time.

In their monograph on the prairie, WEAVER and FITZPATRICK (**14**) stated: "Practically all of the prairies have been mowed annually, some for a period of more than fifty years. It has been repeatedly demonstrated that removal of the plant cover after it is mature has no harmful effect upon the vegetation. After the mowing is done close to the soil surface, there still remains 2.5 to 3 inches (often more on lowland) of unmowed stubble with dead basal leaves, among which accumulates fallen debris during 'hay making,' as well as the late autumnal growth. Undoubtedly the removal of the plants does have an effect not only upon the quantity of organic material that would ultimately return to the soil but also upon the density and composition of the plant cover." The mulch in annually mowed prairies may have a dry weight of 50–225 gm. per square meter on uplands and over 1,000 gm. on lowlands (**15**).

Experiments over a period of only 3–5 years by WEAVER and FITZPATRICK (**14**) have shown that the accumulation of debris greatly retards growth in spring. The soil warms more slowly since it does not receive the usual insolation. Not only is there an actual diminution of basal cover, but also certain of the smaller and earlier species are greatly handicapped in growth and tend to disappear. Thus, occasional fires every 2–3 years renovate unmowed prairies and are distinctly beneficial if they occur in spring before growth is renewed. Fires were undoubtedly frequent in prehistoric times, and the enormous herds of bison trampled down the grass, at least locally.

SAMPSON (**9**), working in the prairies of Illinois, stated: "Occasionally local areas may be found that are seldom disturbed by man. It is in these least disturbed tracts where the dead grass remains from year to year that the dominant plants maintain their purest stand. The secondary species become insignificant, being represented only as scattered individuals here and there. . . ."

CURTIS and PARTCH (**4**), from their experiments on the effect of fire, clipping, and fertilization on transplanted sods of *Andropogon gerardi*, concluded that the most important factor affecting flowering was the presence of old litter on the

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crowns. "Removal of this cover by either fire or clipping brought about a sixfold increase in flower production and a 60% increase in plant height." Where the litter was not removed from dense stands, new growth was delayed in the spring as much as 3–4 weeks. It seems probable that removal of the mulch permitted the plants to begin growth early and to build up a carbohydrate reserve before the normal period of flower primordium initiation (**4**).

An excellent review of the literature on natural mulches of grassland has recently been made by DYKSTERHUIS and SCHMUTZ (**5**). They emphasized the great importance of mulches but pointed out that there has evidently been no earlier research dealing specifically with natural mulches of grassland. "Despite the profound importance of natural mulches in promoting infiltration of rainfall, in prevention of erosion, and in general maintenance of normal plant-soil-water relations, there are virtually no quantitative data concerning amounts or effects on natural grassland" (**5**).

Observations

THE ABANDONED ROAD

During a study of a large native pasture by WEAVER and TOMANEK (**18**) it was found that an abandoned road bounded it on the south. The natural vegetation of this road appeared to be in marked contrast with that of the pastures which adjoined it on both sides as well as with that of an annually mowed native prairie which also extended many rods along its northern border. There was an unusual abundance and rank growth of certain coarser prairie grasses, an almost complete absence of many species normally conspicuous in prairie, and a very deep mulch which had accumulated through several years. The time of abandonment of the road, the absence of fires, and the lack of use of the land for hay or by grazing animals were ascertained from farmers who had lived nearby for 20–50 years. The steeply rolling topography (fig. 1) and the need for upkeep of several culverts were the causes for abandonment 15 years ago.

This road is located 6.5 miles north and 2 miles west of the University of Nebraska in Lincoln. The position of its western one-fourth mile is now marked only by a fence, since the sod was broken, and the soil is under cultivation. The grass on a part of the eastern one-fourth is sometimes cut for hay. It is separated from the central half-mile by a deep ditch. Thus, this part is well protected from disturbance. The road runs along the sides of hills which are 50–100 feet high. The land in the central portion has slopes of 3.5% to more than 17%. Ruts 3–5 inches deep and 12 inches wide still mark the center of this roadway, which is 56.5 feet wide. Sometimes a second track paralleled the original one. The roadsides have probably never been much disturbed since the pastures were fenced many years ago. Exceptions occurred in the central part near two ravines. Here the surface soil had been removed from the steep banks to fill the bottoms of the ravines over the wooden culverts, long since decayed. Sufficient time has elapsed for climax vegetation to be re-established even in these excavated portions and fills. But with these wet areas and banks and the hydric and mesic lowland vegetation which covers them this paper is not concerned. One feature of the upland vegetation, however, should be made clear. Cattle in the rather low-grade pasture south of the road had regularly reached through the fence above a 3-foot, woven-wire base

and grazed the vegetation rather closely in a strip 3–4 feet wide. This vegetation is much like that in the pasture, and only the usual thin mulch common to closely grazed grassland had accumulated here. On the north side dense vegetation continued undisturbed to the fence.

prairie dropseed were intermixed with several other grasses which are often dominant in prairie. These data were obtained by mapping the types as each of several large portions of the abandoned road was laid off in squares 16.5 feet on a side.

FIG. 1.—View looking eastward over rolling hills and dense vegetation in abandoned road, April, 1950. Steel posts on right are 1 rod apart.

COMMUNITIES OR TYPES OF VEGETATION

Examination of the area revealed four types of vegetation, each occurring in at least 85% and often in 98% pure stands. The most extensive was big bluestem (*Andropogon gerardi*). Within this sea of big bluestem occurred islands of prairie dropseed (*Sporobolus heterolepis*), sometimes large but often small. The switchgrass (*Panicum virgatum*) type was interspersed usually in pure or nearly pure stands over certain parts of the area. It was almost always surrounded by big bluestem. A fourth or mixed-grass type occurred in the few remaining places where big bluestem or this species and

Mapping was begun on the east slope of the central hill (fig. 1) and was continued westward over the rather flat top of this hill, a total distance of 17 rods (fig. 2). It was here that much of the experimental work was done. Ten rods of the slope in the foreground (nos. 18–27, fig. 1) were then mapped. A third strip, 10 rods long and west of the part included in the photograph, was selected as representative and also mapped. The big-bluestem community occupied 78% of the mapped area, prairie dropseed and switchgrass 9% and 8%, respectively, and the mixed-grass type was found in only 5%.

Amount and distribution of mulch

In order to ascertain the amount of mulch, samples were taken at random in each type of vegetation. This was done on April 18, 1950. A heavy iron wheel was placed on the mulch, its rim inclosing exactly 5 square feet. With steel clippers the mulch was cut just outside the wheel and separated by pulling it away from the part within. The sample was then removed by hand, using the clippers as needed to cut dead stems of previous years close to the soil. The organic mulch was separated from the mineral soil without difficulty. After the mulch was air dried in the laboratory, it was torn apart and examined, so that no particles of soil or other foreign matter were weighed with the mulch.

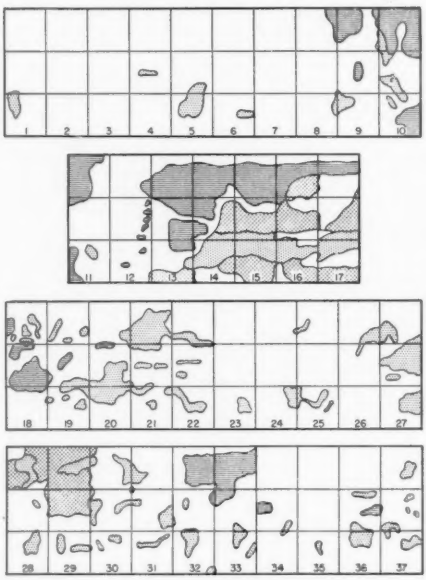


Fig. 2.—Map of representative portions of vegetation in abandoned road. Each minor unit is a square rod. Disturbed vegetation near south fence was not mapped. *Unshaded portion* represents areas occupied by big-bluestem type; *dotted areas*, prairie dropseed; *horizontal lines*, switchgrass; and *cross-hatch*, mixed-grass type.

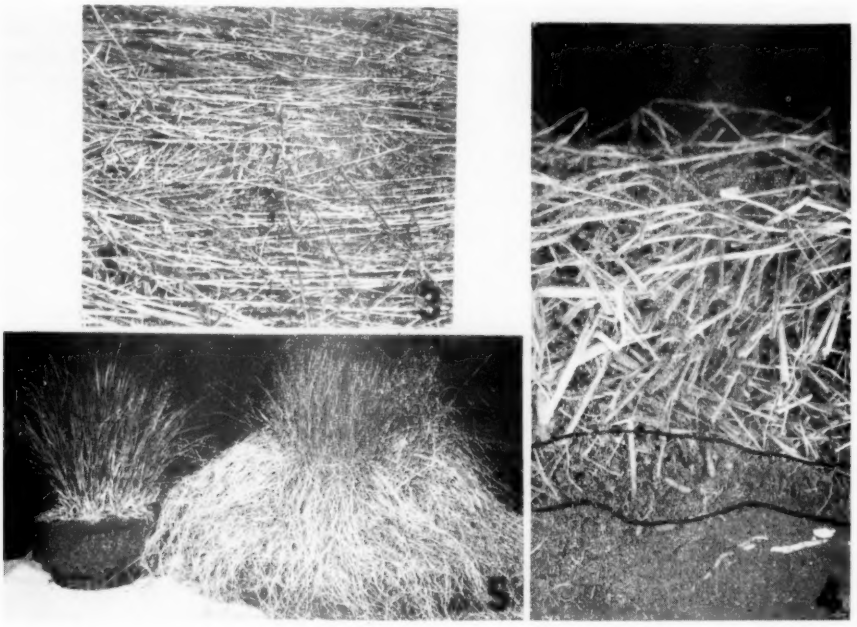
The mulch included all the vegetable matter, in any stage of decay, from the plants of previous years. In the big-bluestem type it occurred only near the soil where the stems, leaves, etc., had fallen to form a layer 5.5–7 inches or more deep (figs. 3, 4). In the prairie-dropseed type, where the bunch had built up a basal column 2–4 inches above the soil, the widely spreading leaves formed a mulch which was thicker between the bunches than just above them. But in the switchgrass type the old stems usually remained standing for at least a year. By clipping the dead plants at the 5-inch level over sample areas of 10 square feet and weighing separately the dried material below and above this level, it was ascertained that approximately half of the total switchgrass mulch consisted of the stems, leaves, and panicles above 5 inches. Attached, dried leaves occurred abundantly only above a height of 2 feet.

There was a thick layer of mulch in all the types of vegetation (table 1). Many other measurements of depth were taken the following year. Rarely was the fallen mulch thinner than 3 inches, and in some places it was 10 inches thick (fig. 5). It reaches a maximum depth in winter and spring, by which time the dead plants of the previous summer had added new materials. By October, before it was thus much augmented, the constant decay of plant materials beneath added much organic matter to the soil. The mulch was then more compacted and thinner. The average amount of well-decayed or humic mulch (5) in the samples in table 1 was 30% by weight in big-bluestem type, and 25, 15, and 39% in switchgrass, prairie dropseed, and the mixed-grass type, respectively.

Soil

The Carrington loam soil is a zonal Prairie soil developed on leached glacial till. It has a dark grayish-brown, granular A horizon 10–12 inches deep, a yellowish-brown, clayey B horizon, which at 3–4 feet in depth gives way to the C horizon of glacial till. Although the calcium carbonate has been leached from the solum, and no free lime is found to a depth of about 5 feet, the pH is usually 6.5. The precipitation of about 27.5 inches annually moistens the soil deeply. Both surface and internal drainage are good.

In the abandoned road there was no evidence of accumulation of dust on the soil surface. The upper 3 inches of soil had a fine-crumb structure—that is, the soil aggregates were soft and porous. Sometimes this structure extended to 4 inches in depth. Below this level the soil had a medium-fine granular structure which was more aggregated. The differences between this soil and that in the adjacent pasture are not in texture (or size of particles) but in structure or composition. The grass roots extended upward clear to the surface of the mineral soil. The coarse roots of big bluestem and switchgrass, which were very abundant, pried the soil particles apart and pro-



Figs. 3–5.—Fig. 3, mulch of big bluestem 8 inches thick covered with fallen flower stalks of previous year. April 18, 1950. Fig. 4, detail of mulch of big bluestem. Slightly decayed upper portion (litter) is 4–5 inches thick; well-decayed friable portion between lines (humic mulch) is about 2 inches deep. These are A_{00} and A_0 horizons as designated by soil scientists. Beneath is mineral soil (A_1 horizon) with rhizomes (white) near surface. Fig. 5, two bunches of prairie dropseed, May 23, 1951. Bases of stems are at same level. Mulch from bunch on left had been removed in early spring.

duced larger openings than did those of the fine-rooted bluegrass and blue grama in the pasture. A vast network of rhizomes also occurred in the surface layer. In the grazed but untrampled strip near the south fence it was found that a thrust by a 175-pound man sank a spade only half as deeply (4–5 inches) as a similar thrust in the heavily mulched portions of the road. The soil appeared even mellower and often more moist under switchgrass than under big bluestem.

TABLE 1

DEPTH OF MULCH (HEIGHT OF ERECT DEAD STEMS OF SWITCHGRASS); AIR-DRY WEIGHT (GM.) PER SAMPLE (5 SQ. FT.); AND CALCULATED TONS PER ACRE. MULCH COLLECTED APRIL 18, 1950

DEPTH (IN.)	DRY WEIGHT (GM.)	TONS PER ACRE
Big-bluestem type		
6.5	931	
6.0	861	
6.0	880	
4.5	751	7.02
7.0	393	
5.5	570	
Prairie-dropseed type		
4.0	847	
5.5	751	
5.5	452	
5.5	724	6.79
4.5	803	
4.5	668	
Switchgrass type		
56	996	
52	934	9.66
65	1,321	
60	772	
Mixed-grass type		
3.5	635	
3.5	717	5.71
4.0	537	
4.5	488	

ORGANIC MATTER AND NITROGEN.—Samples for the determination of organic matter and nitrogen were secured to a depth of 7 inches, after clipping the vegetation at the soil surface and removing all organic mulch. Samples were also taken from an adjacent prairie, of similar southwest exposure and slope, which had been mowed once each year. The percentage of carbon was determined by the SMITH-WELDON method of combustion (**11**) and this was multiplied by the factor 1.72 to obtain the percentage of organic matter. Nitrogen values were obtained by the GUNNING method (**1**). Analyses were made by the Soils Division of the Department of Agronomy, University of Nebraska.

Amounts of both organic matter and nitrogen in the surface soil of the mowed prairie (table 2) are in close agreement with the average from several similar prairies where the samples were taken to a depth of 4 inches (**10**). The organic matter in the surface 1.5 inches of soil under the mulch of bluestem and switchgrass, respectively, exceeded that in mowed prairie by 2.11 and 1.46% of the dry weight of the soil. A similar difference at 1.5–3 inches depth was 0.84% in bluestem; but the amount of organic matter at this level was slightly greater in mowed prairie than in switchgrass. However, at all other depths there was more organic matter in the mulched soil under bluestem. Amounts of nitrogen under the bluestem mulch were 0.095, 0.053, 0.028, and 0.025% of oven-dry weight of soil in excess of those in mowed prairie. The smaller increases under the switchgrass mulch seem to indicate that

this grass is not so effective as big bluestem in enriching the soil.

VOLUME-WEIGHT AND PORE SPACE.—The volume-weight of a soil is the weight of oven-dry soil per unit volume. It is here expressed as grams per cubic centimeter. A volume-weight of 1.12 may be considered as typical for upland virgin prairie soil of medium texture (17). It is low in comparison with that of the second 6 inches (1.31) because of the high degree of granulation and the presence of a large amount of root material in the surface soil. The upper 3-inch layer of soil in the abandoned road under the heavy mulch of big bluestem was so porous that its volume-weight was 0.97. Thus, less than a gram of soil occupied a cubic centimeter of volume.

The volume of prairie topsoil occupied by air and water—the pore space—often exceeds in amount that occupied by solid matter. Pore space is generally greater in fine-textured soils and those of high organic-matter content. It is increased by those processes which promote granulation, such as wetting and drying, freezing and thawing, and particularly the action of the roots and rhizomes of perennial grasses. The pore space in the loose upper 3-inch layer of mineral soil under the mulch was 61.7%. It is believed that this high degree of porosity resulted mainly from the large amount of organic matter and the loosening action of roots and rhizomes, which were abundant even in the surface inch of soil.

EARTHWORMS.—Close examination of the surface soil showed some casts of earthworms. Since practically no earthworms had been observed in removing the mulch or in examining the soil profile, careful studies were made. Early in August, blocks of soil about 6 inches square were removed to a depth of 7 inches. Each of these was placed separately in a half-square-meter pan, the soil was crumbled and worked over thoroughly, and the earthworms counted.

Twenty-one samples beneath the big-bluestem mulch contained only eighteen earthworms. Since one sample had eight and two others two and three, almost two-thirds of the samples had none. Other soil samples to a depth of 12 inches were without worms. The soil was moist but not too wet for good aeration. Its temperature was 71°–72° F. Numerous samples under the mulch of switchgrass gave similar results. In the bluegrass pasture, a few paces beyond the fence, ten samples revealed only seven earthworms.

TABLE 2

PERCENTAGE (OVEN-DRY-WEIGHT BASIS) OF ORGANIC MATTER AND NITROGEN IN SOILS OF ANNUALLY MOWED PRAIRIE AND OF PRAIRIE WITH 15 YEARS' ACCUMULATION OF ORGANIC MULCH

DEPTH (IN.)	% ORGANIC MATTER			% NITROGEN		
	Mowed prairie	Big-bluestem mulch	Switch-grass mulch	Mowed prairie	Big-bluestem mulch	Switch-grass mulch
0.0–1.5	6.21	8.32	7.67	0.303	0.398	0.350
1.5–3.0	4.97	5.81	4.87	.231	.284	.236
3.0–5.0	4.20	4.73	4.20	.198	.226	.199
5.0–7.0	3.65	4.35	3.82	0.180	0.205	0.185

Thus, earthworms were not abundant.

These results are in striking contrast to those of DYKSTERHUIS and SCHMUTZ (5). They found that the surface soil of a relict area of True Prairie near Fort Worth, Texas, unmowed and ungrazed for decades, was covered with a continuous layer of earthworm casts 0.25–1 inch or more in depth. The largest quantity of natural mulch was associated with the greatest accumulation of these aggregates.

EFFECT OF MULCH ON OTHER ENVIRONMENTAL FACTORS

RAINFALL INTERCEPTION.—A cover of grass exerts a profound effect upon the water content of soil. Large amounts of water are held on the plants and evaporated into the air without reaching the soil. For example, CLARK (3) has shown that a thick stand of big bluestem withheld about two-thirds of the precipitation during heavy rains (1.2–1.8 inches) and as much as 97% of very light showers. When an inch of rain falls during an hour, big bluestem with fully developed foliage may intercept over fifty-three tons of water per acre (2). A thick cover of mulch is also capable of causing considerable loss of water to the soil in this manner.

Experiments were made to ascertain how much water was intercepted by the mulch. Places were selected at random in the big-bluestem type. After a small area of the grass had been cut above the mulch and discarded, a circular cardboard, 1 square foot in area was held firmly in place over the mulch. The mulch was then cut with hand clippers around the cardboard to the soil surface and the surrounding mulch pulled away from the sample. Two men with fingers widespread raised the part with intact leaves as a unit from the soil. The humic mulch was also collected, and the entire sample was taken to the laboratory and air dried. Six samples were secured on July 9.

Cylindrical, straight-walled pans, 1 square foot in area and 5 inches deep, with a small hole near the side on the bottom, were used in the experiment. Woven wire with 8 meshes per inch was placed on the bottom of the pan. After wetting the wire and pan and tilting them slightly so that excess water could drain away, the mulch was placed in the pan in its normal position. Water was added to the mulch by means of a spray syringe at a rate of 0.25 surface-inch in 30 minutes. It was added in equal amounts at 5-minute intervals, and the pans were covered between the sprayings. Water not held by the mulch drained into a small funnel placed in a bottle beneath the pan; the insides of both funnel and bottle were wetted before the experiments began. The amount of water intercepted by each of the samples of mulch was 58, 49, 49, 54, 56, and 62%, respectively, of the amount applied. This indicates that an average of more than half of a quarter-inch rain in 30 minutes would not reach the soil. Since no sample of the mulch was found to be thoroughly wet, further studies were made.

The percentage of water intercepted when 0.25 surface-inch was applied each half-hour and the part of a surface-inch held when the mulch was saturated are shown in table 3. The results indicate that in long-continued rains, when the mulch becomes completely wet, as much as one-third inch of rain could be held above the soil and again evaporated into the air. Conversely, the mulch promoted infiltration of water into the soil as was shown experimentally.

INFILTRATION.—Studies were made in midsummer to ascertain the effect of the

mulch upon rate of water penetration. The apparatus and method used were the same as those described by ROBERTSON (**8**). Cylinders made from 15-inch sections of seamless steel tubing 4 inches in diameter and sharpened at one end were forced into the soil vertically with a hydraulic jack to a depth of 14 inches. A coating of light motor oil inside and out, combined with the sharp cutting edge and the thinness of the wall of the cylinder reduced to a minimum the altering of the structure of the inclosed soil (**17**). It seemed desirable to ascertain the rate of percolation in closely adjacent soils which had not been covered with a mulch throughout the 15-year period. Bluegrass pasture occurred on both sides of the road, but here the effect of soil compaction by trampling entered in. Hence, it was decided to use as a control the grazed area just inside the fence where no trampling had occurred. The original bluestem near the fence had been closely grazed so frequently that it had probably been replaced long ago by bluegrass. Here the soil was mellow near the surface but much less so than that under big bluestem. Before the cylinders were sunk, the grass was clipped at a height of 2 inches and removed, and in the big bluestem all but a half-inch of the humic mulch was also removed. A constant head of a few millimeters of water was maintained in each of the four cylinders used in each sampling area. Results are shown in table 4.

TABLE 3

PERCENTAGE OF 0.25 SURFACE-INCH OF WATER HELD BY THE MULCH THE FIRST AND SECOND HALF-HOUR AND TOTAL AMOUNT HELD WHEN SATURATED

Sample	Interception		Water held by saturated mulch (% surface-in.)
	First 30 min. (%)	Second 30 min. (%)	
7	55	12	22
8	50	8	17
9	76	23	32

TABLE 4

AMOUNT OF WATER (CC.) ENTERING THE SOIL IN 30-MINUTE PERIODS IN EACH OF EIGHT CYLINDERS

Half-hour periods	Number of cylinder				Av.
	1	2	3	4	
	Soil under bluegrass				
1st	350	286	500	240	344
2d	15	24	190	10	60
3d	4	38	14	10	16
4th	4	38	15	2	15
	Soil under big bluestem				
1st	670	485	506	346	502
2d	244	20	164	8	109
3d	188	10	146	28	93
4th	188	19	118	23	87

The soil was moist when the experiment was begun. The distance between the cylinders in a group was 4–14 inches, and the distance between the two areas of soil sampled was only 15 feet. Under big bluestem the first 250 cc. of water disappeared in 2–4 minutes in all four cylinders. In two of them a second 250 cc. disappeared within the first half-hour. During the first period the average rate of water infiltration was 46% more rapid under big bluestem. It was 82% more rapid during the second period. During the second hour, rates of infiltration were 481% and 480% greater, respectively, than in bluegrass sod. Thus, once the water reached the soil under the mulch, it entered it rapidly.

EVAPORATION.—The relative rate of loss of water from soil with mulch removed and from that covered with a mulch of big bluestem was ascertained. Four pans, like those previously described but without holes, were used. Potting soil which had been thoroughly mixed was placed in each to a depth of about 2 inches. Equal amounts of water were then added until the soil was very wet and similar to that in the abandoned road in spring. Samples of mulch, cut to fit into the square-foot area of the container, were placed in each of two pans. Two other lots of soil were left unmulched. The pans were placed in a room before a large fan which created a continuous movement of air over them of about 6 miles per hour. Daily temperatures were about 70° F. at night and 80°–95° F. during the day. Humidity varied from 35 to 80%. There was strong diffuse light by day.

Loss of water from the mulched soil during the first day averaged 75% less than that from unmulched soil. It was 43% less the second day. By the end of the third day the bare soil, which had lost a total of 1,187 gm. of water, was dry on the surface and began to crack. The mulched soil had undergone a total loss less than half this great. Losses from the mulched soil decreased gradually as it became drier. Total loss during the 8-day period was practically the same as that during the first 3 days from the unmulched soil.

In a similar experiment (July 26–28) the pans were exposed to both sun and wind from 8:00 A.M. to 7:00 P.M. A wooden frame protected the sides of the containers from the sun. The mulch was 2.5 inches thick in two containers; in the controls the soil was unmulched. Kind of soil and water content were the same as those in the preceding experiment. Both daily and total losses from the mulched soils were very similar. During the first day average water loss from the mulched soil was 74% less than that from the unmulched one, and the loss during the 3-day period was 65% less.

PRECIPITATION AND WATER CONTENT OF SOIL.—During this study there was a good supply of soil moisture for plant growth at all times. Both the amount of precipitation and its frequency of occurrence were unusual. Monthly precipitation from April to September was 4.16, 4.69, 11.94, 3.58, and 4.55 inches. Moreover, the rains were well distributed each month. The soil was wet to moist several feet in depth throughout the growing season. In the spring the total water content ranged from 23 to 27% in the surface foot to 21 to 22% in the second and third foot. In the fourth and fifth foot percentages were 17 and 15, respectively. All but about 9% was available for growth. Probably because of the unusually high rainfall, there were only minor differences in the total amounts of soil moisture in the mulched soil and in soil from which the mulch had been removed in early spring. Water content was also about the same under the grazed but untrampled bluegrass inside the fence (table 5). Soil samples were not

TABLE 5

TOTAL WATER CONTENT OF SOIL AT VARIOUS DEPTHS IN THE ABANDONED ROAD ON AUGUST 6, 1951

(Data from mulched soil are in parentheses)

Community	0–6 in. (%)	6–12 in. (%)	12–24 in. (%)
Big bluestem	22.6 (25.9)	20.9 (19.7)	22.9 (18.8)
Switchgrass	30.7 (37.3)	26.2 (26.9)	23.4 (27.4)
Bluegrass	20.6	21.3	19.2

taken to a greater depth than 2 feet because of great difficulty in withdrawing the geotome from the wet clayey soil.

LIGHT AND TEMPERATURE.—Only 1–5% light penetrated through even the surface inch of the normally compacted mulch. Zero readings were practically always obtained with the Weston Sunlight Meter where it was placed deeper in the mulch. This explains the lack of green color in the basal 2–3 inches of the new shoots.

Soil temperatures at 0.5-inch depth on a clear afternoon, May 7, were 57° and 59° F. under the big-bluestem and switchgrass mulch, respectively, but 79° and 82° in plots with mulch removed in early spring. By May 22, when the air temperature was 71° at 3:00 P.M. on a clear day, temperatures of 89° and 63°, and 90° and 62° were found in the unmulched and mulched soil of big bluestem and switchgrass, respectively. Thus, early in May the upper soil was 22° and 23° colder under the mulch, but by May 22 the mulched soil was 26°–28° colder than that where the mulch had been removed. Readings on July 24 showed only small differences between unmulched and mulched big bluestem. The temperatures in the shade 3 inches above the bare soil, on the soil, and at depths of 1, 3, 6, and 12 inches were 83°, 79°, 75°, 74°, 72°, and 68°, respectively. The temperature was 2° lower over the mulch, 7° lower on the mulched soil surface, and 4°, 3°, 2°, and 0° lower at the several soil depths, respectively. Clearly, the heavy growth of grass had nearly counteracted the effects of heating where the mulch had been removed.

EFFECT OF MULCH ON DEVELOPMENT OF VEGETATION

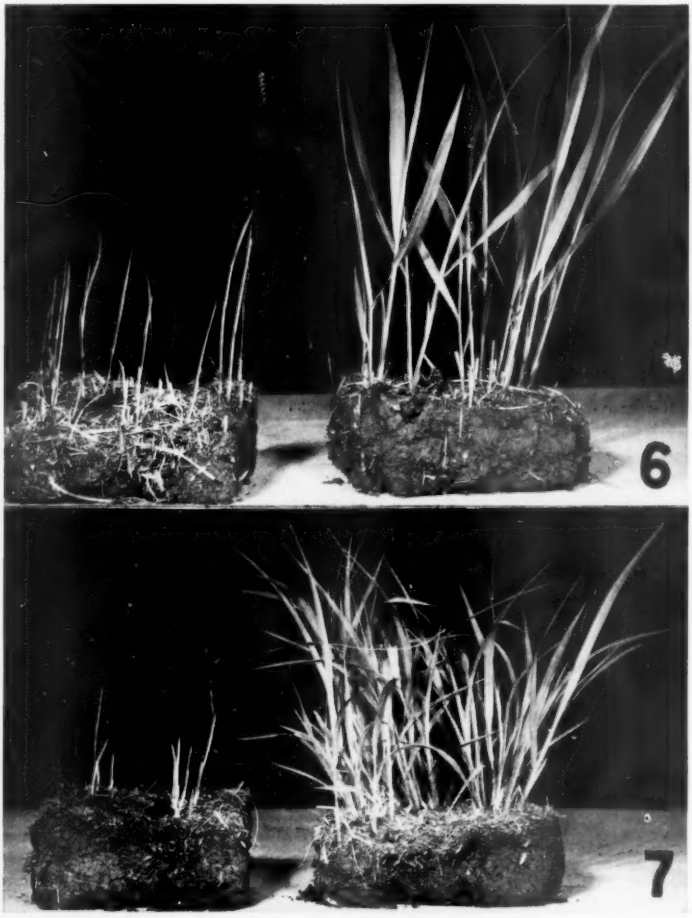
It seemed obvious that the effects of the mulch on the seasonal development of vegetation could best be ascertained by removing the mulch from sample areas and observing the results throughout the summer. On April 7, 1951, two plots in each type of vegetation were freed of their mulch. They will be designated hereafter as unmulched plots. Those in the big-bluestem and switchgrass types extended at right angles to the road or were limited to the upper half of the road and ran parallel with it. They were long plots, 12 feet wide and of sufficient area to furnish 50 square feet of vegetation for each of several harvests. Those in the prairie-dropseed type were smaller, since here comparison of growth was based upon a series of individual bunches. The mulch separated easily from the very moist soil. About one-eighth inch of humic mulch was left to protect the soil from too rapid drying. There was no new growth of vegetation, and any old attached stems were clipped close to the soil. There was no soft, compacted, rotting mass of vegetation. Even the lower part of the mulch was loose, friable, and well aerated.

EARLY GROWTH.—A month later, on May 7, no new shoots had appeared above ground under the mulch, but in the cleared places shoots of big bluestem were 1.5–2.5 inches tall, those of switchgrass 2.5–3.5 inches, and those of prairie dropseed 4.5 inches high.

Fifteen days later the unmulched plants were very leafy at the base. The lower leaves of big bluestem occurred at 1–1.5 inches above the soil. The plants were 6–7 inches tall, and each stem had three or four well-developed leaves. No leaves of the other group had appeared above the mulch. Although the shoots averaged 4 inches in height, practically no leaves were unfolded (figs. 6, 7). Nearly the same stages of development occurred in switchgrass, although the

plants were somewhat taller. The shoots of unmulched prairie dropseed filled both the top and the sides of the elevated crowns almost completely. About 2.5 inches of mulch covered the crowns of the other group, but it was much deeper between the bunches. Here the new shoots were abundant only on the tops of the bunches, and none occurred elsewhere. There were about four leaves per stem on both unmulched and mulched bunches; the latter averaged 2 inches taller than the 8-inch-high, unmulched plants.

EARLY YIELD AND BASAL AREA.—Sample lots of big bluestem and switchgrass were harvested on June 10, each from a 50-square-foot sample in both unmulched and mulched plots. Leaves occurred 1–1.5 inches from the soil on the unmulched big bluestem but at 3–3.5



FIGS. 6–7.—Fig. 6, representative sample of switchgrass from mulched and unmulched plots, May 23 1951. Fig. 7, similar samples of big bluestem.

inches on the mulched plants. On switchgrass these heights were 2–3 and 6–8 inches, respectively, except for the lower, scale-like leaf. After removing the mulch, each lot of grass was clipped to the soil surface. The old stems and debris in the mulched lot were then separated from the green ones of the current year. The green grass was air dried and weighed to the nearest gram. Big bluestem from the mulched plot weighed 53% less than that from the unmulched plot, and switchgrass 57% less. The lower weight must be attributed to the presence of the mulch. Water content of soil was continuously high, but the mulched soil was colder, and the light very low or nil in the mulch.

That the effects of the mulch had greatly thinned the stand was shown both by stem counts and measurement of basal area. In ten square-foot samples taken at random in pure stands of each grass the number of stems under the mulch was 39% lower for big bluestem and 38% less for switchgrass than in unmulched areas. Both grasses were more slender in the mulch.

Basal area was ascertained in a series of thirty-five square-foot random samples in the mulched big bluestem and a similar number in the unmulched plots. The grass was clipped to a height of 5 inches in the mulched samples, and the mulch was removed. Then the plants were clipped to a height of 1.5 inches, as in the plots without mulch, and the basal area at this level was measured. Basal area in the mulched plot was only 9%; but that in the unmulched plot, where the leaves spread widely below the clipping level and stems were also more abundant, was 22%.

Basal area of twelve bunches of unmulched prairie dropseed was ascertained; then the foliage was closely clipped. A dozen mulched bunches of the same basal area were also clipped, and the debris in and about them was discarded. Foliage of the mulched bunches was 2–3 inches taller than the 17-inch general height attained by that of unmulched ones. Stem-counts of several bunches of similar size revealed that there were 18% more stems in those without a mulch. The dry weights of foliage of the twelve unmulched and twelve mulched bunches were 523 and 360 gm., respectively. Weight of foliage from the mulched bunches was thus 31% lower (table 6).

MIDSUMMER YIELDS.—A new lot of plants was clipped on July 15 from areas, each of 50 square feet. Big bluestem had not yet developed flower stalks; those of switchgrass were present in considerable numbers in the unmulched plots, but panicles had not appeared. Flower stalks were fewer and smaller in the mulched area. Heights and yields are shown in table 6. Yields were 26% less in mulched big bluestem and 55% less in mulched switchgrass than in unmulched.

LATE SUMMER YIELDS.—A second lot of bunches of prairie dropseed, eighteen each in the unmulched and mulched areas, was harvested on August 7. These were selected after measurements as at the preceding harvest. Basal area of bunches in each group varied from 20 to 56 square inches. Vegetative growth for the year had been completed. All but five of the unmulched bunches had produced flower stalks; in the mulched lot there were none. A total of 645 panicles were exserted, or nearly so, from the sheath, but some had not yet expanded widely. The unmulched bunches were noticeably more erect than the mulched ones, but the leaves of the mulched plants were longer, and the habit was more sprawling. Thus, the average stretched height in the above sequence was 22 and 25 inches.

When the mulch was discarded and the air-dry plants were weighed, those from the mulched plots weighed 21.5% less than those without mulch (table 6).

On August 16, when the big bluestem in the unmulched area had developed its flower stalks and was in full bloom, another lot of plants was harvested. The foliage height averaged 30 inches and that of the flower stalks 66 inches. Foliage height in the mulched bluestem was 4 inches less, and the scarce flower stalks with inflorescence still ensheathed were usually less than 36 inches tall. Average heights of the unmulched and mulched switchgrass were 62 and 51 inches. Development of the panicles is shown in figure 8. The mulched big bluestem yielded 28.8% less and the mulched switchgrass 25.5% less (table 6) than that from similar areas where the mulch had been removed.

A fourth harvest in September was planned, but heavy rains lodged the grass. This, combined with much cloudy weather, resulted in decay of the lower leaves. It became almost impossible to separate them from the old mulch. Moreover, the plants had completed vegetative growth by mid-August, when deterioration set in. Since only scattered flower stalks were produced, it is probable that the third harvest represented approximately the maximum yield. The area was grazed after September 1.

TABLE 6

AVERAGE HEIGHTS, AIR-DRY WEIGHTS, AND YIELD DIFFERENCES OF GRASSES IN MULCHED AND UNMULCHED PLOTS
(Numbers in parentheses are heights of flower stalks)

Date	Unmulched		Mulched		Yield difference (%)
	Height (in.)	Weight (gm.)	Height (in.)	Weight (gm.)	
	Big bluestem				
June 10	11	1,158	14	550	52.5
July 15	27	3,306	25	2,436	26.2
Aug. 16	30(66)	4,118	26(32)	2,934	28.8
	Switchgrass				
June 10	18	1,057	22	451	57.3
July 15	42	4,168	42	1,898	54.5
Aug. 16	—(62)	6,044	—(51)	4,500	25.5
	Prairie dropseed				
June 10	14	523	16	360	31.2
Aug. 7	18	2,365	16	1,856	21.5

Changes in vegetation

The vegetation in the abandoned road had matured, fallen, and decayed over a period of many years. There was little or

no differentiation in its composition due to the ruts and ridges which, although still prominent, were entirely concealed by the dense stands of grass and the thick carpet of mulch. The nature of the original roadside prairie can be inferred only by comparison with vegetation on similar sites in adjacent prairie on other little-used roadsides. The vegetation was very different from that of mowed prairie, and these differences seem to have been the result of the accumulation of a large amount of debris and its effect upon the environmental conditions.

BIG-BLUESTEM TYPE.—The most extensive type was the rank growth of big bluestem. It seems very probable that this species occurred as an intermixture of 15–18% with other vegetation on the uplands before the 7 years of great drought, 1934–40 (**14**). The much more abundant little bluestem (*Andropogon scoparius*) probably composed 50% of the vegetation, judging from conditions on similar nearby sites on the same slopes and soil type. But little bluestem lost heavily, often 95% of its population, during the drought. It has been widely observed during years of good rainfall (1941–1951) that big bluestem gradually thickened, often to nearly pure stands, and occupied much territory in which, before the drought, it had composed only a small part of the vegetation (**12**). Its great foliage height, 2.0–2.5 feet, and the tall dense stands of flower stalks, its deeper roots, and its sod-forming habit placed the bunch-forming little bluestem, prairie dropseed, and other lower-growing vegetation at a great disadvantage. The large amount of mulch that it produced when it was left ungrazed and unmowed was perhaps as great a detriment to the mid grasses as the shade of the current new growth.

The presence of the debris, both fallen and standing, acted favorably to the retention of snow and its accumulation when blown by the wind. The strip of thick mulch running at right angles to many of the slopes retarded the flow of runoff water from the heavily grazed pasture, while the deep ruts and high ridges

FIG. 8.—Average plants selected to illustrate differences in height and development, August 16, 1951. Big bluestem (*left*) and switchgrass (*right*) with expanded inflorescences are from unmulched plots.

in the old road acted in many places as an effective barrier to water loss. That there was considerable run-in water was shown in a few places where eroded soil had been spread in the upper edge of this belt of tall grass, burying it and permitting the growth of ruderals.

The understory characteristic of upland prairie had all but disappeared. Such low-growing grasses and sedges as *Bouteloua gracilis, B. hirsuta, Carex pennsylvanica,* and *C. meadii* did not occur, and there were only small amounts of *Panicum scribnerianum* and *P. wilcoxianum*—all characteristic plants of upland prairie. Forbs of the lower layer, as *Antennaria campestris, Anemone caroliniana,* and those with rosettes, as *Hieracium longipilum, Senecio plattensis,* and *Erigeron ramosus,* were absent. *Astragalus crassicarpus* and *Sisyrinchium campestre* were rare, and societies of *Erythronium mesochoreum* extremely reduced.

The mid grasses, *Andropogon scoparius, Sporobolus asper, Stipa spartea, Bouteloua curtipendula,* and *Koeleria cristata,* were absent or represented by only a few plants. The only forbs which were of sufficient abundance to form societies of considerable extent were *Amorpha canescens, Aster multiflorus, Ambrosia psilostachya, Artemisia gnaphalodes, Helianthus rigidus, Solidago glaberrima,* and *Rosa pratincola.* Nor did any of these, except the sage, display the flowers to a degree at all comparable with those in mowed prairie. Although about thirty-five other species of forbs were present, none was plentiful.

Not until late June, when viewed from a distance, did the grayish tone of the winter aspect change to one of deep green. Moreover, by early August the aspect was entirely one of grass—the tall, dense stands of big bluestem, with a little Indian grass (*Sorghastrum nutans*) and switchgrass intermixed, overwhelmed other vegetation. In areas of several square yards scarcely another grass or forb occurred. A heavy crop of flower stalks, such as rather regularly occurs on lowlands, was produced in some places in 1949. In the drier summer of 1950 flower stalks were few; nor were they at all abundant in 1951. Switchgrass was adjacent to big bluestem on the moister side and to prairie dropseed on the drier one. But everywhere within its type big bluestem was the sole dominant.

SWITCHGRASS TYPE.—Switchgrass is typically found in habitats that are more moist than those occupied by big bluestem. It is less tolerant of shade than big bluestem, and it does not tiller so readily or so abundantly; but during the first half of the summer it is distinctly taller (**14**). It occurred in dense, pure, or nearly pure stands mostly on the north side of the road where accumulation of snow and, probably, the amount of run-in water were greatest. Where other tall grasses were found, these were sometimes wild rye (*Elymus canadensis*) but mostly big bluestem. It may be that switchgrass is gradually extending its area at the expense of big bluestem. A sprinkling of much attenuated bluegrass and Scribner's panic grass (*Panicum scribnerianum*) stretching up to 2 feet in height, or rarely a stalk of Indian grass, were the only other grasses associated with it. The occurrence of forbs was also greatly restricted. *Rosa pratincola, Amorpha canescens,* and *Solidago glaberrima,* which occurred sparingly, were probably relicts. *Acalypha virginica* occurred commonly and indicated a mesic habitat; *Oxalis stricta* also grew here. But the characteristic forbs were *Galium aparine* and *Vicia americana. Galium* was nearly confined to the habitat of the switchgrass type. It frequently occurred as dense

patches, individual plants reaching a height of 2.0–2.5 feet. *Vicia*, commonly found in lowland or on banks of ravines, began growth early. By the first week in May it was 6 inches above the dense mulch and ultimately attained a height of 2 feet or more. Both species flourished in this shady, moist habitat until midsummer. Other prairie species were excluded from this heavily mulched, upland community. In a few places dense masses of debris had accumulated to a depth of 18–24 inches, so that no vegetation of any kind developed in 1951.

PRAIRIE-DROPSEED TYPE.—The occurrence and present distribution of the much less mesic prairie dropseed has been explained (**12**). It spread down the slopes from the drier upland sites often to the edge of ravines. The myriads of seedlings established during the least severe years of the drought rapidly grew into tufts and then into large bunches. Once a good stand was attained, other grasses and forbs had difficulty in becoming established. In some prairies this spreading continued until 1946.

Where the stand was pure, or even about single large bunches, the mulch was thick; other species were few or none. Heavily mulched spaces unoccupied by any other species were common. Thus, although the somewhat elevated bunches actually covered only 10–15% of the soil surface, other vegetation was largely if not entirely excluded. Three forbs were commonly found in this type—*Amorpha canescens*, *Ambrosia psilostachya*, and *Artemisia gnaphalodes*. *Panicum scribnerianum* was often present, but, in general, well-developed stands of prairie dropseed were conspicuous because of the scarcity of other vegetation.

There is some evidence that big bluestem is replacing prairie dropseed. It has been noted (**12**) that big bluestem, following its recovery from drought, invaded areas occupied by this species, even growing up through the bunches. This tall grass overtopped the dropseed and hindered its growth by shading. The roots of this grass also penetrate deeper than those of the dropseed. The tendency toward more mesic conditions because of the mulch is favorable for this change in the vegetation.

MIXED-GRASS TYPE.—Composition of the vegetation in the mixed-grass community need not be discussed in detail. It probably represents the portion of the original vegetation least modified by complete protection. The mulch was thinner. Here one found not a single dominant grass but many mid-grass dominants, as *Stipa spartea*, *Sporobolus asper*, and *Andropogon scoparius*. There was a small percentage of *Poa pratensis*, *Koeleria cristata*, *Eragrostis spectabilis*, and *Agropyron smithii* in addition to variable amounts of grasses previously mentioned. There were many more species of forbs, and greater numbers as well, than in any of the preceding types. Lower, middle, and upper layers of vegetation were all represented, as were also representative species of each of the several seasonal aspects. This type more nearly approached the norm for True Prairie and thus, by contrast, made clear the changes in the vegetation that had occurred elsewhere.

Discussion

In their study of the prairies and pastures of the loess hills of central Nebraska, WEAVER and BRUNER (**13**) observed the unmowed prairie lying on the steep slopes between the flat-bottomed ravines and the rolling uplands. "On the sides of the ravines above the reach of the mower, the mulch of dead big bluestem was often 5 to 10 inches deep, since the

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prairie was rarely burned intentionally. In this debris, which was sometimes more than a foot deep, one could readily distinguish three crops of dead plants—two of decaying leaves and one of leaf mold—under the living grasses. This accumulation of material prevented the development of an understory, greatly delayed growth in spring, and thinned the new stand of bluestem or other tall grasses to about one-third the usual number of stems."

HOPKINS (**6**), as a result of further study in the same area, stated: "Removal of this mulch in the spring resulted in an increase in basal area by the following autumn of at least threefold. . . . Total mulch had an average air-dry weight of over 15,000 pounds per acre, and as much as 19,000 pounds was present where conditions permitted the best development of vegetation. . . . This represented an accumulation of the growth of several seasons. . . . The surface of the soil was always moist beneath the mulch even in the driest weather. Where the prairie was mowed annually there was an average of only 838 pounds of mulch [per acre] on the soil surface."

Amount of mulch in a relict area of climax prairie near Fort Worth, Texas, which had been unmowed and ungrazed for decades, was compared by DYKSTERHUIS and SCHMUTZ (**5**) with that from a native hay meadow, on comparable soil. The meadow had been ungrazed for many years but mowed once annually in July. Both mulch and green herbage were obtained from three meter quadrats in each of the two grasslands. The mowed grassland yielded 1,938 pounds of air-dry organic mulch per acre; the undisturbed grassland had accumulated 9,037 pounds. The green herbage from the mowed prairie, however, was much greater (1,033 pounds) than that from the deeply mulched prairie (621 pounds). Thus, a decrease in forage production was associated with the heavier mulch.

Measurements of yield in the 290-acre pasture adjacent to the abandoned road gave similar results in 1950. Because of poor distribution of the grazing animals in this 50-year-old prairie, about 12% of the area had been grazed so lightly that a dense mulch, 3–5 inches thick, had accumulated. In this portion not only was growth in spring greatly delayed and various species (including Kentucky bluegrass) more or less excluded but also the seasonal yield was considerably lower (**18**). This decrease was verified by further (unpublished) studies in 1951. Usually, however, increase in mulch is one of the indicators of potentially higher yields.

In drier climates an organic mulch may not accumulate in grassland in excessive amounts, even if the cover is undisturbed. For example, LARSON and WHITMAN (**7**), working in the Mixed Prairie of southwestern South Dakota, reported forage yields which varied directly with the depth of the natural mulch. But here the virgin grassland, long protected from grazing and fires, had accumulated throughout the years a mulch which was only 0.25–0.5 inch deep.

Summary

1. An upland prairie, undisturbed by mowing, grazing, or burning for 15 years, was studied. About 80% was covered with a nearly pure stand of *Andropogon gerardi*. Similar stands of *Panicum virgatum* and *Sporobolus heterolepis* each occupied about 9%.

2. A dense natural mulch, 4.5–8 inches deep and weighing 6–9 tons per acre, covered the deep loam soil. The mulch inter-

cepted much precipitation but promoted more rapid infiltration of water which reached the soil and greatly retarded evaporation. It increased the organic matter of the surface soil 1.5–2% and nitrogen content 0.1% of its oven-dry weight.

3. Roots and rhizomes grew thickly below the mulch and mellowed the surface soil. Pore space was 61.7%, and volume-weight was 0.97.

4. Soil temperatures 22°–28° F. lower under the mulch in May delayed growth about 3 weeks compared with plants where the mulch had been removed. Production of flower stalks was considerably later in all mulched grasses and also less in *Andropogon*. Flower stalks were abundant on all unmulched grasses.

5. Yields in June, July, and August were 53, 26, and 29% less from mulched stands of *Andropogon*, and 57, 55, and 26% less in *Panicum* than those from unmulched stands.

6. Consequences of the effects of the mulch upon the environment were the production of a nearly pure, but somewhat thinner than normal, stand of *Andropogon*. The understory characteristic of upland prairie had all but disappeared. The usual mid grasses of upland were few or none. Only a few of the taller forbs remained.

7. Dense stands of *Panicum* with forbs characteristic of very moist sites were typical. Between the large bunches of prairie dropseed the deeply mulched soil was usually free of vegetation.

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EMBRYOLOGY OF MACROSOLEN COCHINCHINENSIS

P. MAHESHWARI AND BAHADUR SINGH

Introduction

The literature on the embryology of the Loranthaceae has been reviewed by SINGH (**15**) in his paper on *Dendrophthoe falcata*. The only other recent work is that of MAHESHWARI and JOHRI (**11**) on *Helixanthera ligustrina* (Wall.) Dans., which confirms SCHAEPPI and STEINDL's (**14**) statement that the upper part of the embryo sac in *Helixanthera* grows into the style and reaches the stigma.

The embryology of *Macrosolen* has been studied by TREUB (**16**), who described *Loranthus sphaerocarpus* Bl. (*Macrosolen cochinchinensis* Van Tieghem), and by SCHAEPPI and STEINDL (**14**), who have studied three species—*M. cochinchinensis*, *M. formosus*, and *M. pseudoperfoliatus*—paying special attention to the first. Our observations on *M. cochinchinensis*, which are more complete than those either of *Treub* or of SCHAEPPI and STEINDL, are presented in this paper.

Material and methods

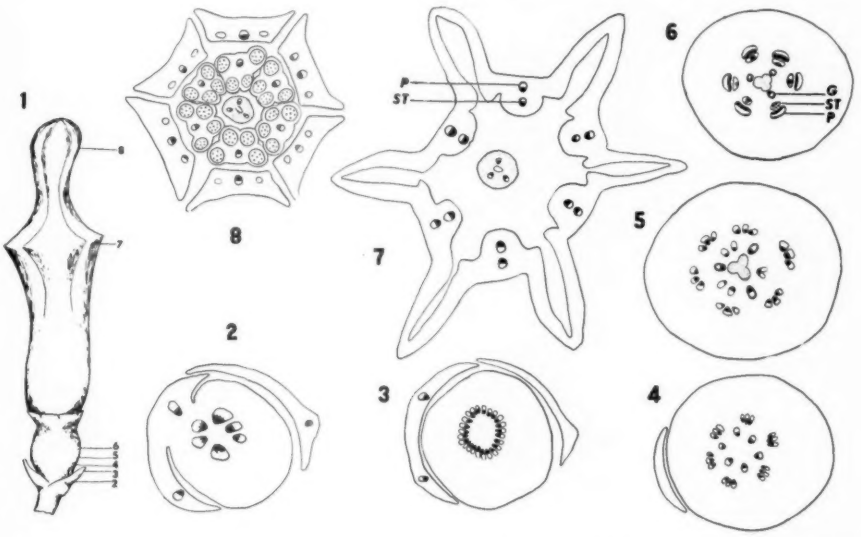
A fairly extensive collection of buds, flowers, and fruits of *M. cochinchinensis* was made during 1941–1944 in the vicinity of Dacca in eastern Bengal. Some material was also obtained from Cuttack. Formalin-acetic-alcohol and Navaschin's fluid were used for fixation. Of these, the former proved more satisfactory. The sections were stained with Heidenhain's iron-haematoxylin and safranin-fast green. Permanent acetocarmine smears of the microspore mother cells were made, using MCCLINTOCK's method (**8**).

Observations

FLOWER.—The yellowish-red flower possesses one bract and two bracteoles. It has an infundibuliform green calyculus which is devoid of any vascular supply. The perianth tube consists of six members. In a fully grown bud (fig. 1) the tube is narrow in the lower part, widens near the middle, then narrows again, and terminates in a club-shaped apex. As the flower opens, the lobes of the perianth become strongly reflexed above the swollen region. There are six epiphyllous stamens, one opposite each perianth lobe. The anther is approximately 0.5 mm. long, and its inner lobes are slightly longer than the outer. In the bud the anthers are closely pressed against the style and attain a level just below the stigma. They are syngenesious but become separated where the flower opens. A long narrow style with a swollen conical base and a hollow stylar canal arises from the center of the ovary. From the base of the ovary arises a nipple-shaped process or "mamelon," which terminates near the place of attachment of the style (figs. 18, 19, 21).

After supplying the bracteoles, the stalk shows a ring of six vascular bundles (fig. 2) which divide into a much larger number at the base of the flower (fig. 3). Slightly higher up they become arranged in two rings of which the inner contains six bundles, and the outer, eighteen, arranged in six groups of three, each alternating with the bundles of the inner ring (fig. 4). At the level of the mamelon (fig. 5), which apparently lacks a vascular

supply, three of the inner bundles are opposite the lobes of the mamelon, and the other three are opposite the notches. The former enter the style and undergo some further divisions near the stigma. The remaining three bundles bifurcate to form the six staminal traces. The eighteen outer bundles fuse in groups of three to form six bundles, one entering each perianth lobe (figs. 5–7). The staminal bundles continue undivided into the connective, but the perianth traces undergo some branching and anastomosing so that each lobe may have three to five strands (fig. 8).

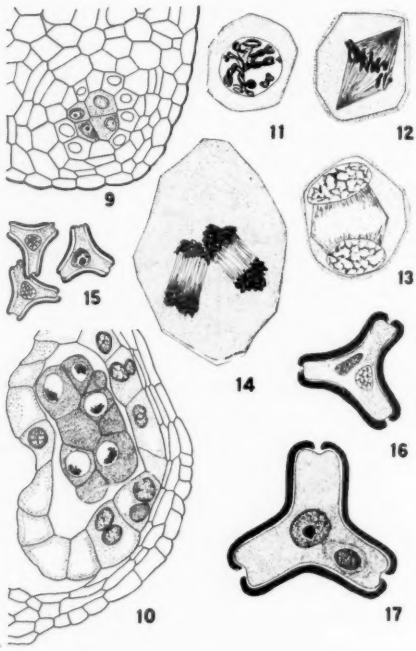


FIGS. 1–8.—Vascular supply of flower. Fig. 1, diagram of flower bud (×2.1) to show levels at which cross-sections shown in figures 2–8 (×14) have been cut. *G*, stylar trace; *P*, perianth trace; *ST*, staminal trace; *stippled area*, mamelon.

MICROSPOROGENESIS.—A cross-section of the young anther shows a group of microspore mother cells surrounded by tapetum, middle layer, endothecium, and epidermis (fig. 9). The microspore mother cells are polygonal with a large nucleus (fig. 10). At diplotene (fig. 11) each chromosome is formed of two chromatids. The tetrads become much shorter and thicker at diakinesis, then the nuclear membrane disappears, and the nucleus passes into the first metaphase (fig. 12), followed by anaphase and telophase (fig. 13). There is scarcely any interkinesis, and the second reduction division sets in very quickly (fig. 14), resulting in a tetranucleate mother cell. Cytokinesis takes place by furrowing, and the tetrads are of the tetrahedral or of the decussate type, as in *Dendrophthoe* (**15**).

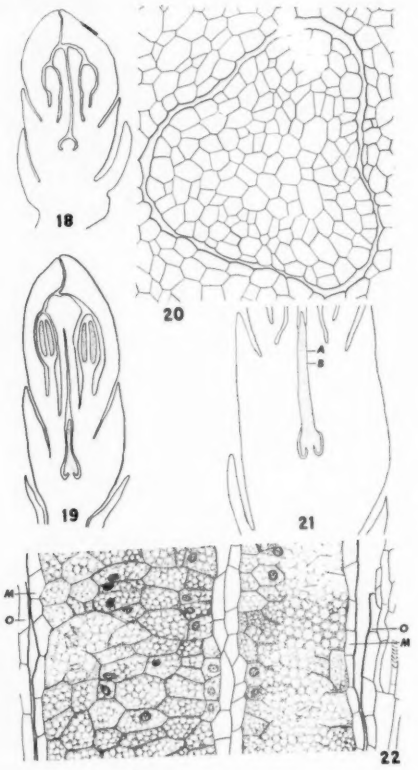
MALE GAMETOPHYTE.—The microspores acquire a three-rayed outline (fig. 15), as in other members of the Loranthoideae (**14**). The exine is uniformly thick except at the three pores, where only the intine is present. The nucleus of a microspore divides to give rise to the tube and generative cells. The generative nucleus is more or less elliptical (fig. 16), and the cytoplasm surrounding it stains

more lightly. The vegetative nucleus is spherical and somewhat larger than the generative (fig. 17). The endothecium had not developed any fibrous thickenings, and the tapetum was still recognizable in anthers which showed two-celled pollen grains. In this connection the tapetum in *Scurrula* begins to disintegrate at the time of the second meiotic division, while in *Dendrophthoe* it remains intact until the time of formation of the microspore tetrads (12). In *Macrosolen* it appears to persist still longer.



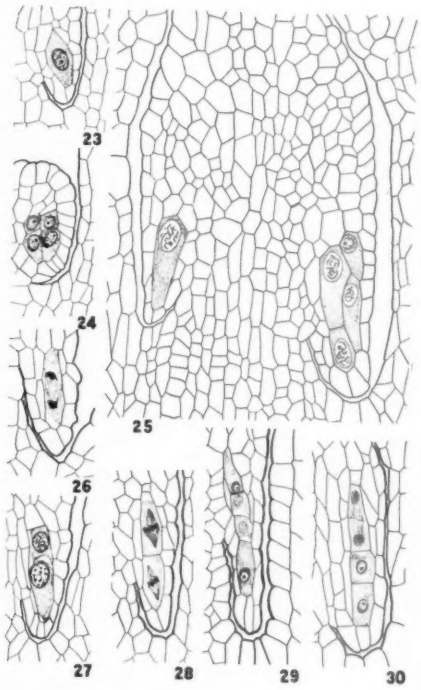
FIGS. 9–17.—Stages in microsporogenesis and development of male gametophyte. Figs. 9, 10, 15, ×287; figs. 11, 12, 13, 16, ×625; figs. 14, 17, ×781. Fig. 9, transverse section of part of anther at microspore-mother-cell stage; fig. 10, same, showing mother cells in synizesis; fig. 11, mother cell showing diplotene stage; fig. 12, metaphase of meiosis I; fig. 13, end of meiosis I; fig. 14, telophase of meiosis II; note obliquely placed spindles; fig. 15, microspores showing intine, exine, and three germ pores; fig. 16, young bicelled pollen grain; fig. 17, mature bicelled pollen grain; generative cell has moved inward from original position near wall.

OVARY.—Fig. 18 shows a longitudinal section of a young flower bud. The small mound-shaped projection arising from the base of the ovary is the mamelon, which elongates upward and finally reaches the base of the style (figs. 19, 21). It is three-lobed at the base (fig. 20),



FIGS. 18–22.—Mamelon development. Figs. 18, 19, 10, 21, ×11; fig. 20, ×33; fig. 22, ×208. Fig. 18, longisection of young bud showing mamelon arising from base of ovary; fig. 19, slightly older stage showing upward elongation of mamelon; fig. 20, cross-section through lower part of mamelon showing trilobed nature; fig. 21, longisection of ovary showing maximum height obtained by mamelon; fig. 22, magnified view of part of mamelon between levels *A* and *B* in figure 21 (*O*, ovary wall; *M*, epidermis of mamelon). The walls of some cells are so thin as to be almost unrecognizable.

each lobe representing a downward-facing ovule which projects into the cavity of the ovary. The central portion of the mamelon consists of narrow, elongated cells, but the cells of the outer region are large, polygonal, and highly vacuolate. Their walls are extremely thin and hardly recognizable at many places. The epidermis of the mamelon, which is formed of narrow, elongated cells, is closely pressed against the wall of the ovary (fig. 22).



FIGS. 23–30.—Stages in megasporogenesis. Figs. 23, 24, ×222; figs. 25–30, ×208. Fig. 23, longisection through one lobe of mamelon showing hypodermal archesporial cell; fig. 24, group of four archesporial cells in one lobe of mamelon; fig. 25, longisection of lower part of mamelon showing one megaspore mother cell in left lobe and four in right; fig. 26, megaspore mother cell in telophase; fig. 27, dyad; fig. 28, nuclei of both dyad cells in metaphase; fig. 29, linear tetrad of megaspores; fig. 30, delayed division of upper dyad cell.

MEGASPOROGENESIS.—An archesporium of one to four cells originates in the hypodermal layer of each of the three lobes of the mamelon and is distinguishable from surrounding cells by larger cell size and denser cytoplasm (figs. 23, 24). The archesporial cells function directly as megaspore mother cells. They elongate (figs. 25, 26) and sometimes become spindle-shaped as do those of *Dendrophthoe falcata* (**15**). They undergo the usual reduction divisions (figs. 26–28) to form linear tetrads of megaspores (fig. 29). Frequently two such tetrads are seen in a lobe (fig. 31), and sometimes as many as three may be present. Rarely the division of the upper (the words "upper" and "lower" are used throughout the paper in the sense of position in the ovary and have no relation to the micropyle or chalaza unless so indicated) dyad cell was delayed (fig. 30).

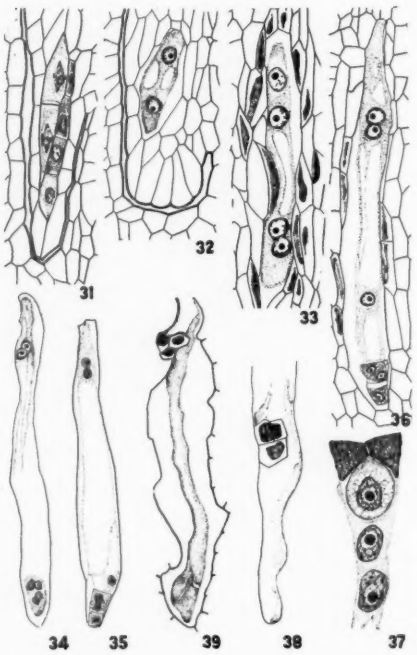
As a rule, the basal megaspore—the one situated toward the epidermis—functions. Occasionally it is the subbasal which enlarges, while the basal and the upper two megaspores begin to degenerate. The degenerating megaspores may persist even up to the two- or four-nucleate stage of the embryo sac.

EMBRYO SAC.—Figure 32 shows a two-nucleate embryo sac. Soon after the division to form four nuclei the embryo sac elongates considerably, and those cells of the mamelon which lie in its path are crushed and destroyed (fig. 33).

All four nuclei of the embryo sac do not appear to divide simultaneously, for the next stage shows a six-nucleate condition in which four nuclei lie at the lower end and only two at the upper end (fig. 34). For a time the tip of the embryo sac continues to grow upward, leaving the two nuclei behind. Three of the nuclei at the lower end soon become delimited as antipodal cells, and the

fourth or lower polar nucleus begins to travel upward (figs. 35, 36).

In the eight-nucleate embryo sac the upper end (fig. 37) lies in the apex of the mamelon slightly below the base of the style. In this respect *Macrosolen* is simpler than some other members of the Loranthaceae—such as *Dendrophthoe*, in which the egg apparatus comes to lie about two-thirds of the way up the stylar canal, and *Helixanthera*, in which it extends up to the stigma.



FIGS. 31–39.—Embryo-sac development. Figs. 31, 36, 37, 38, ×214; figs. 32, 33, ×263; figs. 34, 35, ×129; fig. 39, ×180. Fig. 31, one megaspore tetrad and two dyad cells in division; upper three megaspores of tetrad are in process of degeneration, basal megaspore enlarging; fig. 32, binucleate embryo sac; fig. 33, four-nucleate embryo sac; note degeneration of surrounding cells of mamelon; fig. 34, six-nucleate embryo sac formed by division of two basal nuclei of four-nucleate stage; fig. 35, same, with three antipodal cells organized; fig. 36, older stage showing upward movement of lower polar nucleus; fig. 37, upper end of mature embryo sac showing degenerating synergids, egg, and two polar nuclei; fig. 38, lower end of embryo sac showing downwardly directed process or caecum; degenerated antipodals remain in situ; fig. 39, same, slightly later stage.

The synergids as well as the antipodal cells are ephemeral. In the mature embryo sac the polar nuclei are very prominent and always lie just below the egg.

From the base of the sac arises a broad caecum or haustorium which elongates downward leaving the antipodal cells in situ (figs. 38, 39), but it was not clear whether it passes through the stalk of the mamelon or enters directly into the base of the ovary.

At this time the parenchymatous cells lying in the basal part of the ovary become filled with food material. They are surrounded by a few layers of thick-walled, feebly collenchymatous cells which are laid down more or less in the form of a test tube (figs. 44–50). In conformity with other authors we have designated these cells as collenchymatous, but in later stages their walls become thickened more or less uniformly and also show signs of lignification.

EMBRYO.—The fertilized egg begins to elongate downward. The first division is longitudinal (fig. 42), as in the other members of the family so far investigated, and probably occurs about the same time as the first division of the endosperm, but this point could not be definitely ascertained. The second division is transverse (fig. 43) and is followed by further transverse divisions resulting in a long biseriate proembryo which grows down through the endosperm tissue (fig. 44). By the time a proembryo has reached the base of the endosperm, the embryo-sac caecum has already entered the tissue inclosed by the tube of collenchyma cells. Although three to four embryos are present in an ovary, only one of them is generally able to reach

the bottom of the tube (figs. 45, 46). This one grows more vigorously, while the others are crushed and absorbed.[1] The suspensor, which is greatly coiled and twisted, remains biseriate throughout its length except near the embryo proper, where it becomes thicker as the result of a few longitudinal divisions.

Although further downward growth of the embryo is stopped by the collenchyma cells, coiling of the suspensor cells apparently continues so that the embryo is pulled back up into the endosperm, leaving an empty space in the collenchyma tube (fig. 47).

ENDOSPERM.—Triple fusion was observed to occur as in other angiosperms. The primary endosperm nucleus is formed just below the egg but soon travels downward and comes to lie in the base of the caecum. The first division of the endosperm mother cell is followed by a transverse wall (fig. 41). The second division is also transverse. Further divisions could not be followed very closely, but those in the upper half of the embryo sac are mainly transverse, and even the mature endosperm consists of only about four vertical rows of cells. In the lower half, divisions take place in all directions to form a much broader tissue, which becomes filled with large quantities of food material.

When the proembryos have grown down into the lower halves of their respective embryo sacs, the individual endosperms fuse to form a composite mass (figs. 47, 48), as has been described for *Dendrophthoe* (**15**).[2] This composite endosperm grows vigorously, and the cells of the mamelon are soon consumed by it. At the same time, its lower part broadens and extends around the collenchyma tube, which is present at the base of the ovary (figs. 49, 50).

FRUIT.—The fruit is a globose pseudoberry, containing a single embryo imbedded within a massive endosperm. A seed-coat is absent, and the endosperm is surrounded directly by the pericarp, which has a complicated structure resembling that reported for *Dendrophthoe falcata* (**15**).

The two cotyledons of the embryo, one frequently larger than the other, point downward. The small inconspicuous plumule lies between them. The radicle end of the embryo is large and massive and projects above the endosperm.

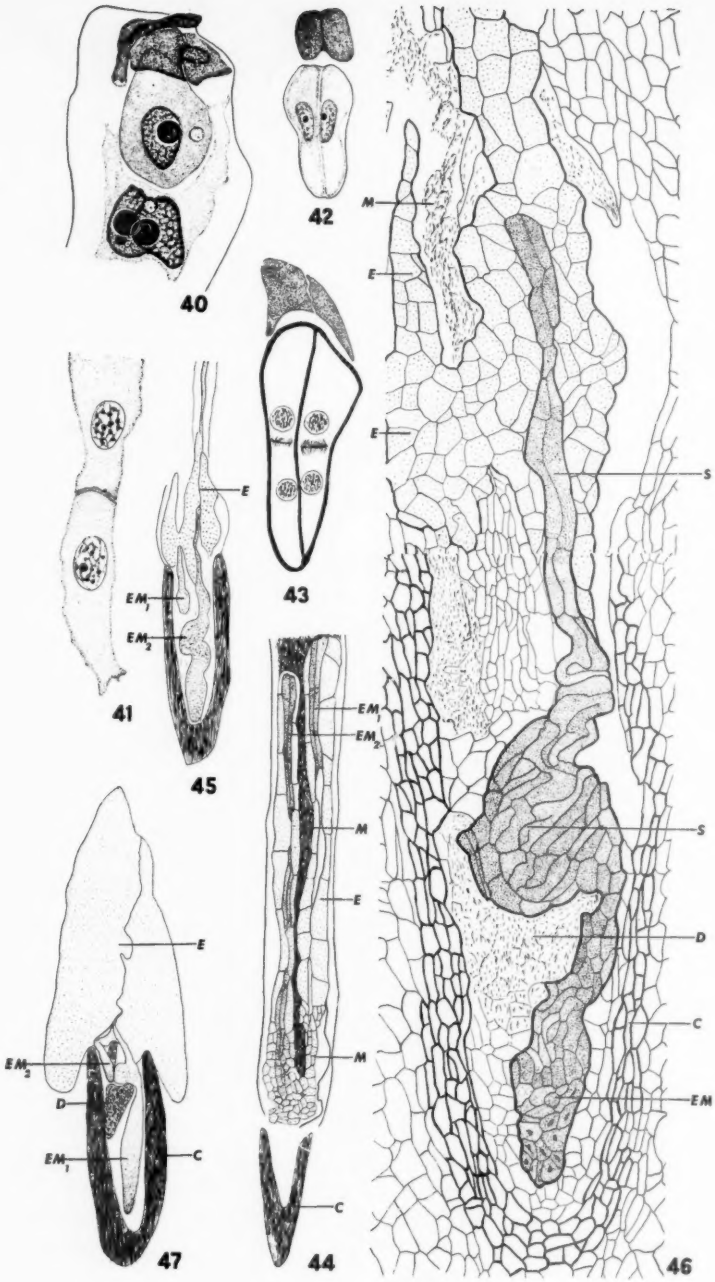
The basal portion of the endosperm is not flat but deeply concave, projecting upward. This is due to the endosperm having grown down around the tube of collenchyma which persists in the seed. The upper part of the endosperm is open like a flower vase with six conical teeth. In the cavity of the vase is found the embryo, with its radicular end showing above the teeth of the endosperm (figs. 52, 53).

Histologically, the endosperm shows three somewhat different regions (fig. 51). There is an outer layer of small empty cells; the innermost layers are more or less flattened and crushed as a result of the growth of the embryo, while the central region consists of polygonal cells, many of which show degenerated nuclei. A degeneration of nuclei in mature endosperm cells has also been recorded in several other plants (**10**).

The pericarp is composed of four zones of cells. The inner zone, next to the endosperm, consists of elongated and crushed

[1] In *Loranthus*, GRIFFITH (**4, 5**) mentioned the possibility of a fusion of several embryos to form one embryo. Although this is not impossible, such a case was not found here in *Macrosolen cochinchinensis*.

[2] It may be noted that as early as 1844 GRIFFITH (**5**) observed, in *Loranthus*, "the formation of one mass of albumen from the albumina of several ovula." He was, however, incorrect in saying that the albumen may even develop outside the embryo sac.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FIGS. 40–47

parenchymatous cells, with the vascular bundles running through it. As stated earlier, the vascular bundles entering the base of the ovary (fig. 54) resolve themselves into two rings: the inner, of six bundles; the outer, of eighteen bundles in six groups of three each (figs. 55, 56). At the points where the inner vascular bundles are situated, the outward growth of the endosperm becomes checked so that a cross-section of the latter gives a furrowed appearance, the furrows lying opposite to the vascular bundles (figs. 50, 55, 56). At about one-third of the height of the fruit wall the inner bundles become pushed out and come into line with the outer bundles. Above this the growth of the endosperm is quite uniform, and a cross-section through it presents a circular outline (fig. 57). In the upper part of the fruit, where the endosperm becomes six-lobed, each lobe is surrounded by the vascular traces which entered the floral organs (figs. 58–60).

Outside the vascular region is a narrow zone of three to four layers of rather large parenchymatous cells having prominent vacuoles.

Next comes the viscid layer, extremely prominent, which is responsible for the ejection of the ripe seed from the fruit. The cells in this zone are greatly elongated and extremely thin-walled. They are curved like the spirals of a wire brush used for cleaning rifles. In the basal part they form a collar around the vascular tissue entering the ovary.

The outer part of the fruit wall has a somewhat leathery texture as in *Dendrophthoe* but lacks the stone cells found in the latter. It contains three somewhat differentiated regions. Immediately outside the viscid layer are two or three layers of slightly elongated, thin-walled cells followed by four to six layers of larger cells which are often filled with a densely staining material, while the epidermis consists of very small cells.

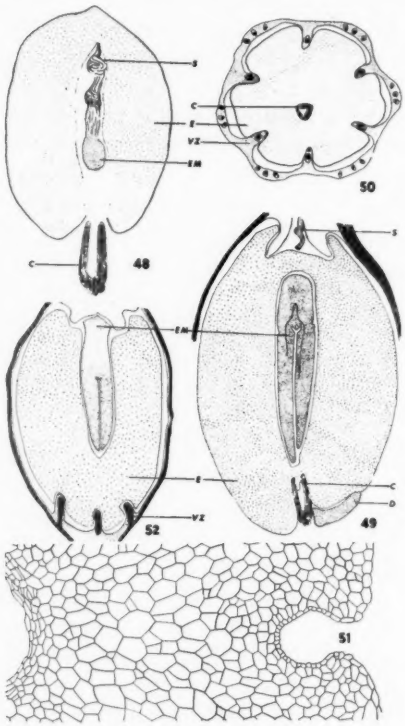
Discussion

It may be mentioned in discussing the observations on *Macrosolen* that a mamelon is also found in several other genera of the Loranthaceae, namely, *Lepeostegeres*, *Elytranthe*, and *Amyema*. It is, however, absent in *Dendrophthoe*, *Scurrula*, *Helixanthera*, *Taxillus*, and *Barathranthus* (**14**).

The morphological nature of the mamelon has long been a subject of controversy. GRIFFITH (**5**) suggested that it is to be regarded as a placenta, and this view was supported by TREUB (**16, 17,**

FIGS. 40–47.—Stages in development of embryo and endosperm (*C*, collenchyma; *D*, degenerated cells of ovary inclosed in collenchyma tube; *E*, endosperm; *EM*, embryo; *M*, degenerated cells of mamelon; *S*. suspensor). Fig. 40, ×523; figs. 41, 43, ×321; figs. 42, 46, ×262; fig. 44, ×42; fig. 45, ×14; fig. 47, ×26. Fig. 40, upper end of embryo sac showing double fertilization; fig. 41, lower part of caecum showing division of primary endosperm nucleus to form two cells; fig. 42, bicelled proembryo; first division of egg is longitudinal; note degenerated remains of synergids; fig. 43, four-celled proembryo; fig. 44, longisection of central part of ovary showing two embryo sacs in which proembryos (EM_1 and EM_2) have grown down to nearly the base of endosperm (*E*); owing to their tortuous path proembryos cannot be seen from end to end in a single section; fig. 45, embryos have pierced endosperm and one of them has reached base of collenchyma tube; fig. 46, embryo and surrounding tissues shown more highly magnified than in figure 45; fig. 47, embryo receding from base of collenchyma tube; endosperms of all the embryo sacs have more or less fused to form one composite mass.

18). HOFMEISTER (7), on the other hand, regarded it as equivalent to a single erect ovule. DOWDING (1), on the basis of work on *Arceuthobium americanum*, considered that the mamelon represents a second whorl of carpels. She accepted the theory of carpel polymorphism propounded by SAUNDERS and interpreted the gynaecium of *Arceuthobium* as composed of two alternating whorls of four carpels each.



FIGS. 48–52.—Later stages in development of embryo and endosperm (*C*, collenchyma; *E*, endosperm; *EM*, embryo; *S*, suspensor; *VZ*, inner wall of ovary containing vascular bundles). Figs. 48, 52, ×6.8; figs. 49, 50, ×9; fig. 51, ×102. Fig. 48, longisection of endosperm and embryo; note coiled suspensor; fig. 49, older stage showing endosperm growing down around tube of collenchyma (patch of cells marked *D* belongs to ovary and will be consumed by endosperm); fig. 50, transverse section of endosperm showing furrowed outline; note inner wall of ovary *VZ* containing vascular bundles, and collenchyma tube *C;* fig. 51, transverse section of strip of endosperm passing through a furrow; fig. 52, longisection of seed showing embryo projecting above endosperm.

The most recent detailed work is that of SCHAEPPI and STEINDL (13, 14), who agreed with GRIFFITH and TREUB in regarding the mamelon of the Santalaceae and Loranthaceae as a "Samentrager" or placenta which is similar to the free central placenta of the Primulaceae. The projections or lobes formed on the mamelon must then represent the ovules. We are in agreement with this view. It follows that, in those genera in which a mamelon is absent, reduction has gone so far that the embryo sacs arise directly in the tissues at the base of the ovary.

The lobes of the mamelon of *Macrosolen*, which we have interpreted as ovules, do not show any differentiation into nucellus and integument. There is therefore nothing which can be designated as the micropyle, but, if the analogy with *Thesium* (2) is correct, it is the side toward which the hypodermal archesporium differentiates which must be considered as micropylar. Since a comparative study of several other members of the Loranthaceae is in progress in our laboratory, further consideration of this point will be postponed for the present.

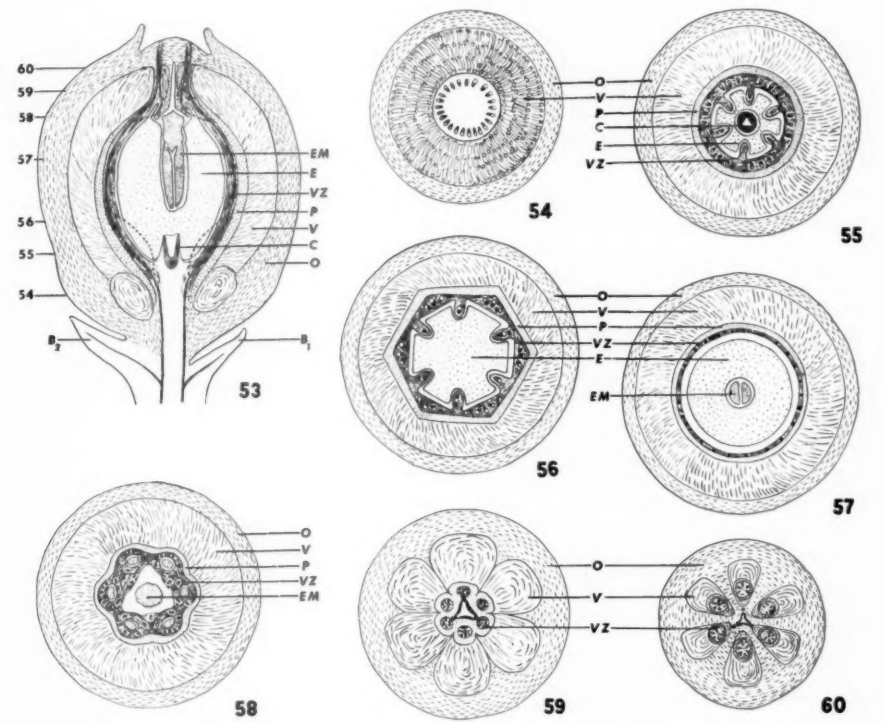
TREUB (16) reported that in *Macrosolen cochinchinensis* the megaspore mother cell forms a row of three cells. SCHAEPPI and STEINDL (14) were in agreement with this, although they explained that one of the cells of the "triad" is binucleate. Our observations differ from those of these authors, for, although wall formation may be delayed in the upper dyad cell, undoubted linear tetrads are formed.

TREUB (16) and SCHAEPPI and STEINDL (14) further stated that the upper-

most cell of the triad (the one toward the style) functions. We have found this to be incorrect. It is the basal cell which enlarges and gives rise to the embryo sac, while the upper three cells soon degenerate and disappear.

During further development, according to TREUB, one division is omitted in the basal end of the embryo sac, so that a three-nucleate embryo sac is formed in place of the usual four-nucleate stage. SCHAEPPI and STEINDL made no categorical statement on this point but mentioned having seen undoubted four-nucleate embryo sacs in *Macrosolen pseudoperfoliatus* and once also in *M. cochinchinensis*. The occurrence of a four-nucleate stage was found to be a normal feature of our material.

TREUB and SCHAEPPI and STEINDL failed to see any antipodal cells. In the present investigation several embryo sacs were found with three distinct antipodal cells. Careful study of SCHAEPPI and STEINDL's statements and figures disclosed that they had misinterpreted the antipodal cells as the three nonfunctioning megaspores. For example, their figure 18*a*, interpreted as an embryo sac with two degenerating megaspores at the base (one megaspore considered binucleate), represents, in our opinion, a six-nucleate



FIGS. 53–60.—Diagrammatic sections of fruit. Fig. 53, median longisection showing levels at which various cross-sections represented in figures 54–60 have been taken (*B*, bracteole; *C*, collenchyma tube; *E*, endosperm; *EM*, embryo; *O*, outer wall of fruit; *P*, zone of parenchyma; *V*, viscid layer; *VZ*, vascular zone).

stage with two nuclei at the upper end and the three antipodal cells and lower polar nucleus at the basal end. Certain of TREUB's figures (Plate X, figs. 5, 6) must also be interpreted as six-nucleate embryo sacs. It appears probable that he missed one of the nuclei in these embryo sacs. The same reinterpretation may apply to SCHAEPPI and STEINDL's figure 28*b* of *Elytranthe parasitica;* hence it may be considered to represent the antipodal end of an embryo sac rather than a megaspore with its three degenerating sister cells. Attention is also called to a misinterpretation of the opposite nature made in the case of the Onagraceae, where the three nonfunctional megaspores at the chalazal end were long mistaken for antipodal cells (see **9**).

A new feature is the formation of an embryo-sac caecum which elongates downward and touches the bottom of the collenchyma tube at the base of the ovary, leaving the antipodal cells in situ. The formation of such a caecum is common in several members of the Centrospermales and in certain other plants. (**10**) but has evidently not been recorded previously in the Loranthaceae. It is probable that the formation of the caecum and the consequent relegation of the antipodal cells to one side explain the infrequency with which the latter have been observed in the Loranthoideae.

Endosperm formation begins in the basal end of the caecum and gradually extends toward the upper part of the embryo sac. In this respect the Loranthoideae seem to differ from most other angiosperms, and the subsequent fusion of the individual endosperms of the different embryo sacs to form one composite structure is another feature which is peculiar to the members of this tribe.

A longitudinal division of the zygote is common to all members of the Loranthaceae so far studied and has also been reported in *Balanophora* (**19**) and *Langsdorffia* (**3**). A peculiarity of the Loranthoideae is the extreme elongation of the suspensor, which pushes the embryo proper from the place of zygote formation[3] into the basal part of the endosperm. In *Macrosolen* the embryo pierces the endosperm and encroaches upon the ovarian cells lying inside the collenchyma tube. Its further growth is stopped by the collenchyma, but meanwhile the suspensor cells above become coiled and twisted like a ball of yarn, so that the embryo is retracted into the endosperm.

We believe that members of the Loranthoideae are without a parallel in the complexity of their embryo sac, endosperm, and embryo; further studies of a thorough and detailed type are needed to clear up some of the obscurities of their embryology. In addition, there are other unsolved problems, such as the morphological nature of the calyculus, the structure of the gynaecium, and the presence or absence of an integument. Considerable comparative study of the morphology of the Loranthaceae, Balanophoraceae, and Santalaceae will be necessary before any definite opinions can be put forward on these problems. Another family showing abnormalities of a somewhat similar nature is the Monimiaceae (**6**), but work on this is even more meager than on the families belonging to the order Balanophorales.

Summary

1. The flower of *Macrosolen* is hexamerous. There is an infundibuliform

[3] In *Lepeostegeres*, fertilization of the egg occurs at a point approximately one-quarter the height of the style; in *Amyema*, somewhere near its middle region; in *Taxillus* and *Dendrophthoe*, slightly above the middle; and in *Helixanthera*, just below the papillate layer of the stigma (**11**, **14**, **15**).

calyculus, a long perianth tube which opens at the tip into six highly reflexed lobes, and six epiphyllous stamens. The ovary is inferior, and from its base there arises a trilobed projection, the mamelon, which reaches up to the junction of the ovary and the style.

2. The vascular supply of the flower is based on a hexamerous plan. The perianth tube receives six vascular bundles, each of which divides to form three to five strands. The stamens receive one vascular bundle each, and three bundles enter the style.

3. The anther wall is formed of the epidermis, an endothecium, one middle layer, and a single-layered tapetum composed of binucleate cells.

4. The microspore mother cells divide simultaneously and form tetrads of the tetrahedral or decussate type.

5. The pollen grains are shed in the two-celled condition.

6. There is a one- to four-celled archesporium which arises in the hypodermal layer of each of the three lobes of the mamelon.

7. Linear tetrads of megaspores are formed. The basal megaspore generally functions. Development proceeds normally up to the four-nucleate stage of the embryo sac.

8. All four nuclei do not divide simultaneously. The two basal nuclei divide first, so that a six-nucleate stage intervenes before formation of the final eight-nucleate stage. The three antipodal cells are organized at the six-nucleate stage. The synergids are ephemeral, but their degenerated remains persist even up to formation of the four-celled proembryo or later.

9. During maturation of the embryo sac the cells at the base of the ovary just below the mamelon develop collenchymatous thickenings. They are laid down in the form of a deep cup or test tube, which is filled with thin-walled cells rich in food material.

10. The upper end of the embryo sac comes to lie in the apex of the mamelon. From the lower end a caecum or lateral haustorium grows into the tube of collenchyma, while the antipodal cells degenerate in situ.

11. Double fertilization occurs normally. Triple fusion takes place just below the egg.

12. The first division of the zygote is longitudinal and is followed by a series of transverse divisions resulting in a biseriate proembryo with an extremely elongated suspensor which causes the embryo to pierce the endosperm and enter the tube of collenchyma.

13. Generally only one of the three embryos formed in an ovary reaches down to the bottom of the tube. This embryo alone reaches maturity, while the others are crushed and absorbed.

14. Soon after touching the bottom of the collenchyma tube the embryo is retracted into the endosperm by the coiling of the suspensor.

15. The mature embryo possesses two free cotyledons, a small conical plumule, and a swollen radicle.

16. The endosperm is cellular, and the endosperms of all the embryo sacs fuse to form a composite mass which grows vigorously and consumes the mamelon. At its upper end it becomes separated into six toothlike structures.

17. The seed is naked, and the endosperm lies directly within the fruit wall, which is formed of four regions of cells. As in other members of the Loranthoideae the viscid layer is very well developed and is responsible for ejection of the seed and its adhesion to the substrate.

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DEPARTMENT OF BOTANY
UNIVERSITY OF DELHI
DELHI 8, INDIA

DEPARTMENT OF BOTANY
B.R. COLLEGE
AGRA, INDIA

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STUDIES OF FLORAL MORPHOLOGY IN THE ERICALES
II. MEGASPOROGENESIS AND MEGAGAMETOPHYTE DEVELOPMENT IN THE ANDROMEDEAE[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 634

BARBARA F. PALSER

Introduction

A consideration of the vascular anatomy and organography of the flower in the Andromedeae (**23**) (part of a comprehensive morphological study of the flower of the Ericales which was undertaken to help arrive at answers to certain phylogenetic and taxonomic problems) has led to a tentative arrangement for twenty-two species of the tribe. The slides used in that study to determine the pathways of the vascular strands also showed some stages in the development of the ovule. As additional buds of different ages had been collected for several of these species, these were sectioned, and the present paper describes the development of the ovule up to the mature megagametophyte stages in fifteen of the twenty-two species.

Of the twelve embryological characters which MAHESHWARI (**21**) listed as of major taxonomic significance in helping to delimit the larger plant groups, five are covered by the observations reported in the present paper: (*a*) development and structure of the ovule, (*b*) form and extent of the nucellus, (*c*) origin and extent of sporogenous tissue in the ovule, (*d*) megasporogenesis and development of the megagametophyte, and (*e*) the form and organization of the mature gametophyte. Variations with respect to these features and others of less significance should serve to corroborate, or make less tenable, the relationships suggested in the earlier paper.

Several reports on some stages of ovule development have been made for certain species of the Ericales. PELTRISOT (**25**) and ARTOPOEUS (**1**) were primarily interested in the development of the seed but in some instances briefly described the mature gametophyte. SAMUELSSON (**26**) for a variety of species, STEVENS (**27**) for *Epigaea*, BEIJERINCK (**3**) for *Calluna*, and OLIVER (**22**) for *Sarcodes* gave a little more detail on developmental stages. More recently COPELAND for the Rhododendroideae (**6, 14**), Monotropoideae (**6, 7, 8, 10–13**), Pyroleae (**6, 15**), and *Pholisma* (**9**); DOYEL and GOSS (**18**) for *Sarcodes;* DOYEL (**17**) for *Arctostaphylos viscida;* KAVALJIAN (**19**) for *Clethra alnifolia;* and CHOU (**5**) for three species of *Gaultheria* have supplied additional details. No paper has been found, however, which reports the complete development of several relatively closely related species or which makes comparison between species within a relatively small group, such as a tribe.

Material and methods

The collections of material and the names used for the various species are the same as in the first paper of this series (**23**) and are given in table 1 of that paper. Some additional collections

[1] This work was supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

have been made: *Cassiope tetragona* by PAUL D. VOTH at Point Barrow, Alaska; *Epigaea repens* by ALMA G. STOKEY on Mount Holyoke, Massachusetts, by G. NORMAN PALSER on the outskirts of Worcester, Massachusetts, by JOHN R. RAPER in Great Smoky Mountains National Park, and by WILLIAM H. HATHEWAY at various stations in Harvard Forest, Massachusetts; and of several species by myself at places similar to those already listed. All species studied previously have been observed, at least in the mature megagametophyte stage, and several followed in some detail: *Andromeda glaucophylla*, *A. polifolia*, *Cassiope mertensiana*, *Chamaedaphne calyculata*, *Enkianthus cernuus*, *Epigaea repens*, *Leucothoe axillaris*, *L. catesbaei*, *L. recurva*, *Lyonia ligustrina*, *L. mariana*, *Neopieris nitida*, *Oxydendrum arboreum*, *Pieris floribunda*, and *Xolisma fruticosa*.

Additional sections cut particularly for this study were 10 μ in thickness and either cross or longitudinal, depending upon the orientation of the ovules in the particular species. In many species the orientation of the ovules is not regular, so that relatively few sections give a good picture of gametophytic structure. Most of these slides were stained with Heidenhain's iron-alum hematoxylin and counterstained with Orange G.

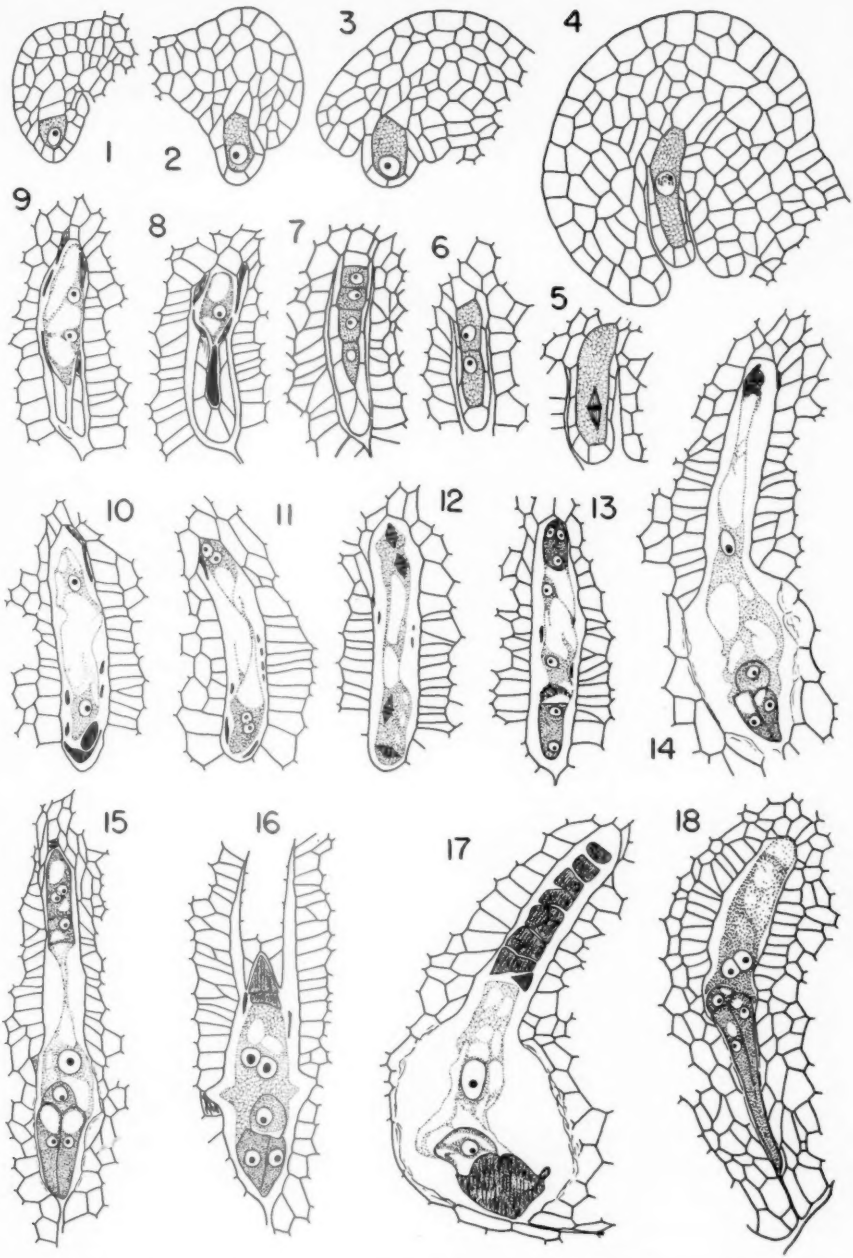
All drawings were made with a camera lucida at uniform magnification for comparable sections; in those instances in which some of the gametophytic nuclei occurred in adjacent sections, these were added to make a composite drawing. Because of its peculiarities, development in *Cassiope mertensiana* was photographed rather than drawn.

Observations

There is considerable similarity in the various species, indicative of their close relationship, so that a generalized description is possible. Certain variations do occur, however, and the descriptions for each species essentially point out only its peculiarities.

GENERAL.—The size of the mature ovule varies considerably within the Andromedeae (fig. 63), and its condition varies from almost strictly anatropous (fig. 63, Leu. cat.) to campylotropous (fig. 63, Leu. rac.). In every case, however, the ovule has a single integument, the outer layer of which is made up of cells which, except for those close to the micropyle, are filled with a substance probably tannin-like in nature. The inner layer of the integument differentiates, in the area surrounding the nucellus, into a more or less clearly defined layer of cells termed "endothelium" by MAHESHWARI (**21**), that is, narrow cells with the long axis perpendicular to the gametophyte. The nucellus is a single layer in thickness and disintegrates fairly early in the development of the megagametophyte, so that the latter comes in direct contact with the integument. After disintegration of the nucellus the gametophyte projects anteriorly into the micropyle, where it may bulge considerably at the expense of integumentary cells. The endothelium, developed only around the nucellus, thus surrounds just the chalazal end of the gametophyte.

In development the ovule is initiated as a small protuberance on the placenta. This ovular primordium soon begins to bend, and at about this time the archesporium is differentiated (figs. 1, 36). The initiation of the integument follows, beginning with divisions, periclinal and then anticlinal, in a few epidermal cells near the outside of the bend (figs. 2, 36). The integument increases in size and develops laterally until, at the megaspore-mother-cell stage, it has developed



FIGS. 1–18.—Figs. 1–14, *Andromeda glaucophylla*, megasporogenesis and development of megagametophyte. Fig. 15, *Leucothoe catesbaei*, mature megagametophyte. Fig. 16, *Enkianthus cernuus*, mature megagametophyte. Fig. 17, *Leucothoe racemosa*, mature megagametophyte. Fig. 18, *Epigaea repens*, essentially mature megagametophyte; many show a secondary nucleus rather than two polars and have sharp "ears" which this section does not show.

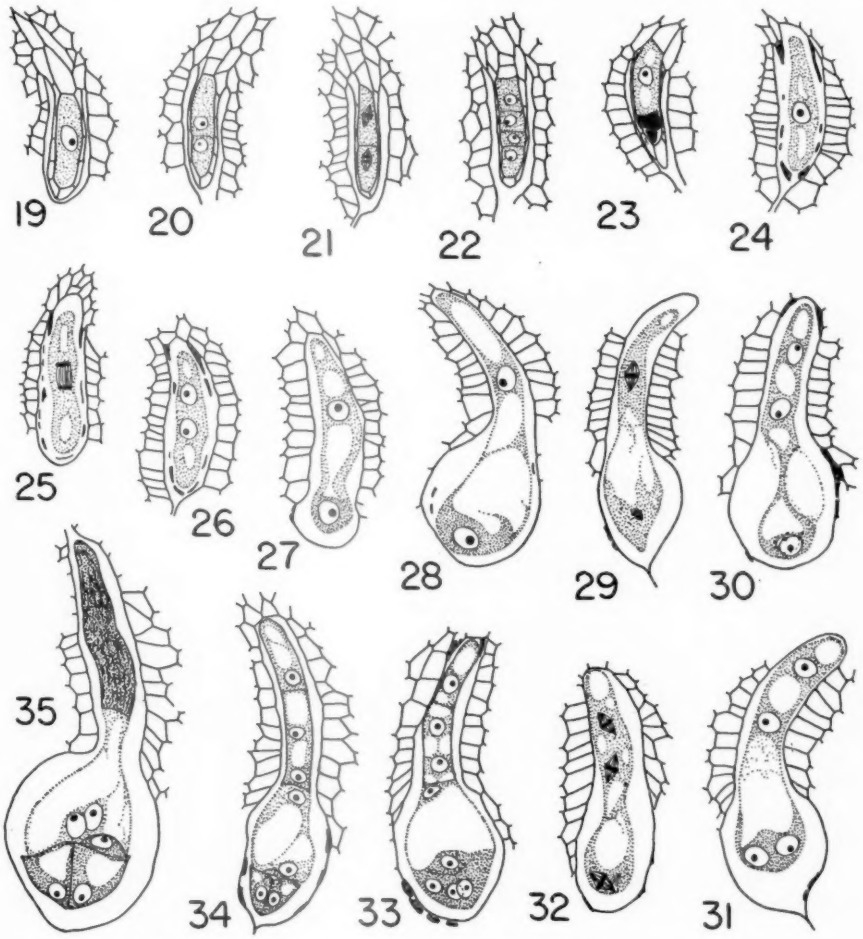
to a point almost parallel with the tip of the nucellus (figs. 4, 37). Subsequently it completely surrounds the latter, and the bend continues until the ovule is essentially anatropous (or campylotropous). Funiculus and integument are undiverged from each other throughout almost their entire length on the inside of the bend.

A single hypodermal cell forms the archesporium, and this gives rise directly to the megaspore mother cell (figs. 1–4). In all but one of the species studied in detail (*Cassiope mertensiana*) the megaspore mother cell undergoes meiosis to give rise to a linear row of four megaspore cells (figs. 4–7, 19–22), of which normally the three micropylar ones disintegrate and the chalazal one enlarges and functions in forming the megagametophyte (figs. 8, 23). In two species the micropylar dyad cell occasionally fails to divide, thus giving rise to a linear row of three cells of which the micropylar two disintegrate. Three successive mitotic divisions give rise to the eight nuclei which form the mature megagametophyte (figs. 9–13, 25–33). Development, therefore, follows the Polygonum Type (21).

Vacuolization starts in the functional megaspore where vacuoles develop at both ends of the cell and the nucleus is centrally located (figs. 8, 23, 24). In the two-nucleate stage the nuclei occupy the ends of the gametophyte, and a single large vacuole is located centrally (figs. 10–12, 27–33), a condition which persists to the early eight-nucleate stage. The eight nuclei organize a seven-celled structure (figs. 14–18, 35): two synergids, one egg, one endosperm precursor with two polar nuclei (or one secondary nucleus resulting from the fusion of the two polars), and three antipodals. Typically the egg apparatus is contained in a broadened area anterior to the endothelial layer of the integument, while the polar nuclei (or secondary nucleus) occur at just about the level of the micropylar end of the endothelial layer, and the antipodal cells are in the narrow region surrounded by it. The polars may or may not fuse prior to fertilization, depending upon the species. The size and fate of the antipodal cells are also variable. In some species they are quite large (figs. 15, 35) and in others small (fig. 14); they may persist until after fertilization or may completely disintegrate prior to maturity of the megagametophyte (fig. 49). In two species there is a multiplication of antipodal tissue (fig. 17). Size and shape of the synergids are somewhat variable; in one species in particular they are extremely elongated and penetrate relatively close to the external opening of the micropyle (fig. 18). Two species show peculiar, well-developed "ears" projecting laterally just posterior to the egg apparatus (fig. 16). One species, and to a much lesser extent a second, has a "pedestal" of nucellar tissue between the mature megagametophyte and the region of contiguity of the nucellus with the integument (fig. 16).

AMPELOTHAMNUS PHYLLIREIFOLIUS.—The mature ovule is fairly small, slightly campylotropous, the integument about six cell layers in thickness, and the endothelial layer rather weakly developed (fig. 63, Amp.). Developmental stages were not available for this species. In the mature megagametophyte the region containing the egg apparatus is somewhat expanded, the synergids are slightly elongated, and the antipodals, rather large and distinct, are usually linearly arranged. The polar nuclei are generally fused by the time the gametophyte is mature.

ANDROMEDA GLAUCOPHYLLA.—The mature ovule is very large and essentially campylotropous. The integument is about ten cell layers in thickness, and the endothelial layer clearly differentiated (fig. 63, And. glauc.). The complete developmental story is available in this species (figs. 1–14). Because M. E. BRITTON of Northwestern University made collections for me at weekly intervals in a single location in southern Michigan, the approximate rate of development is also known. The ovule is differentiated during the fall of the year prior to the opening of the flower. It overwinters in an early archesporial-cell stage with the integument not yet developed. The archesporial cell begins to enlarge and the integument is initiated during the end of March or early in April and reaches the megaspore-mother-cell stage in the middle of April. This im-



FIGS. 19–35.—*Chamaedaphne calyculata.* Megasporogenesis and development of megagametophyte.

mediately undergoes meiosis to form the linear tetrad of megaspores. The micropylar spores disintegrate, and the functional chalazal spore enlarges and divides rather quickly so that a week later a few early two-nucleate stages may be found. The end of the month finds the gametophytic divisions and enlargement being completed, and the gametophyte is mature after the first week in May. Enlargement is gradual through all gametophytic stages until the early eight-nucleate stage. Considerable elongation occurs between this stage and the mature gametophyte. The nucellus begins disintegration at the chalazal end during the tetrad stage, and a few cells may be distinct at the micropylar end until the early two-nucleate stage, but in subsequent stages only occasional disintegrating remains are found. The mature gametophyte is elongated, the micropylar end somewhat enlarged. The synergids are rather small, the polar nuclei may or may not be fused, and the antipodal cells are small and partially disintegrated.

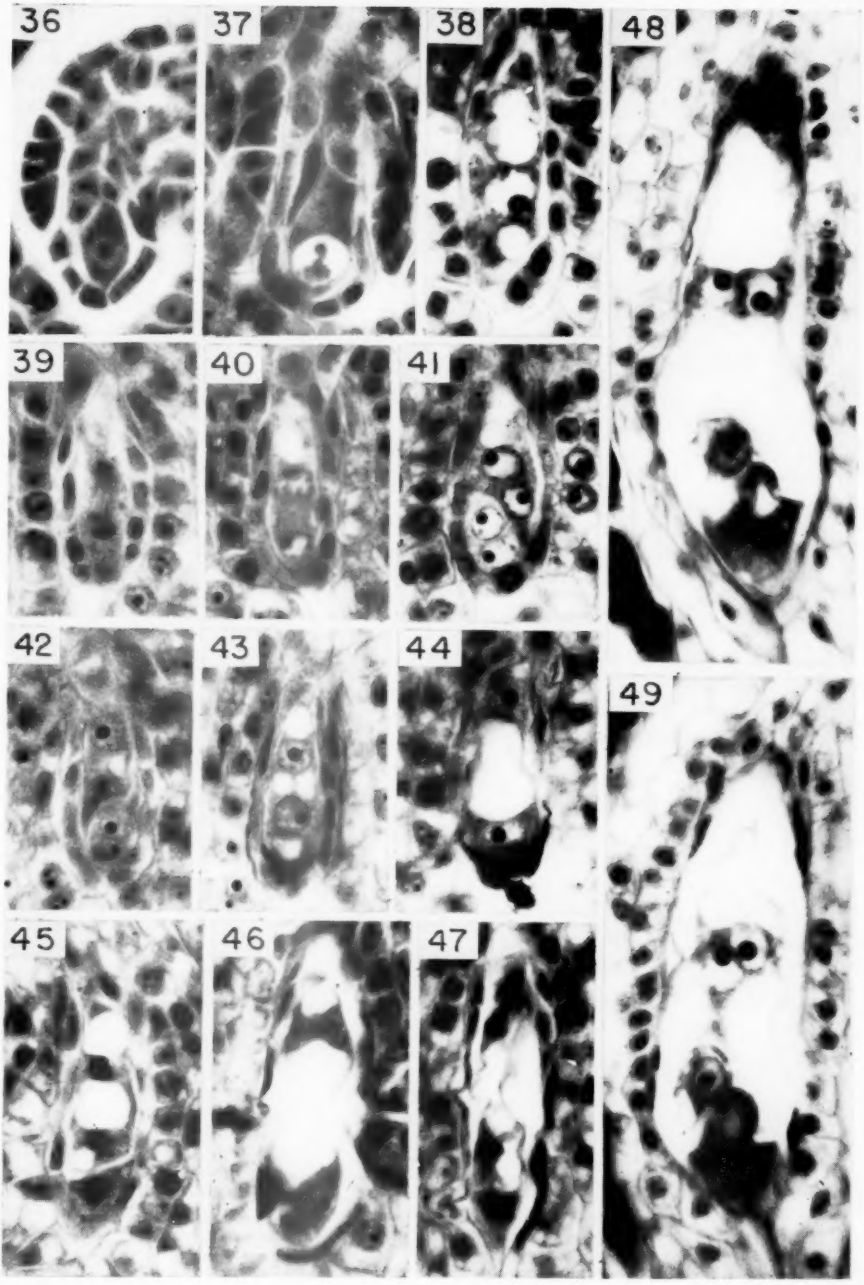
ANDROMEDA POLIFOLIA.—The characteristics of the ovule and development of the gametophyte in this species are closely similar to those in *A. glaucophylla*, but the time sequence has not been followed. The ovule is somewhat smaller with an integument eight cell layers in thickness (fig. 63, And. pol.).

CASSIOPE HYPNOIDES.—Each ovule in this species is small, essentially anatropous, the integument about four cell layers in thickness and the endothelium rather clear (fig. 63, Cas. hyp.). No stages of development between the archesporial cell and the disintegrating mature gametophyte are available at the present time. The only mature gametophytes are those unfertilized ones present in material which showed the early developmental stages of endosperm and embryo (**24**) and so appear partially disintegrated. The whole gametophyte is relatively broad, the micropylar end only slightly more so than the mid-region. The antipodal end may be narrowed. The synergids are broad, the polar nuclei apparently fuse, and the antipodal cells can be distinguished, though they are small and largely disintegrated.

CASSIOPE LYCOPODIOIDES.—The mature ovule is similar to those of *C. mertensiana* and *C. tetragona:* small, essentially anatropous, the integument approximately four cell layers in thickness, and the endothelium slightly differentiated (fig. 63, Cas. lyc.). Nothing earlier than the mature gametophyte has been found in the material available. This shows little or no micropylar enlargement; the synergids frequently show a distinctive bulge on the chalazal end which contains a vacuole; the polar nuclei occasionally fuse, and normally the antipodals have completely disintegrated by the time the gametophyte is mature. One ovule showed two gametophytes, one of which was partially disintegrated. This may perhaps suggest that development in this species is similar to that described below for *C. mertensiana*.

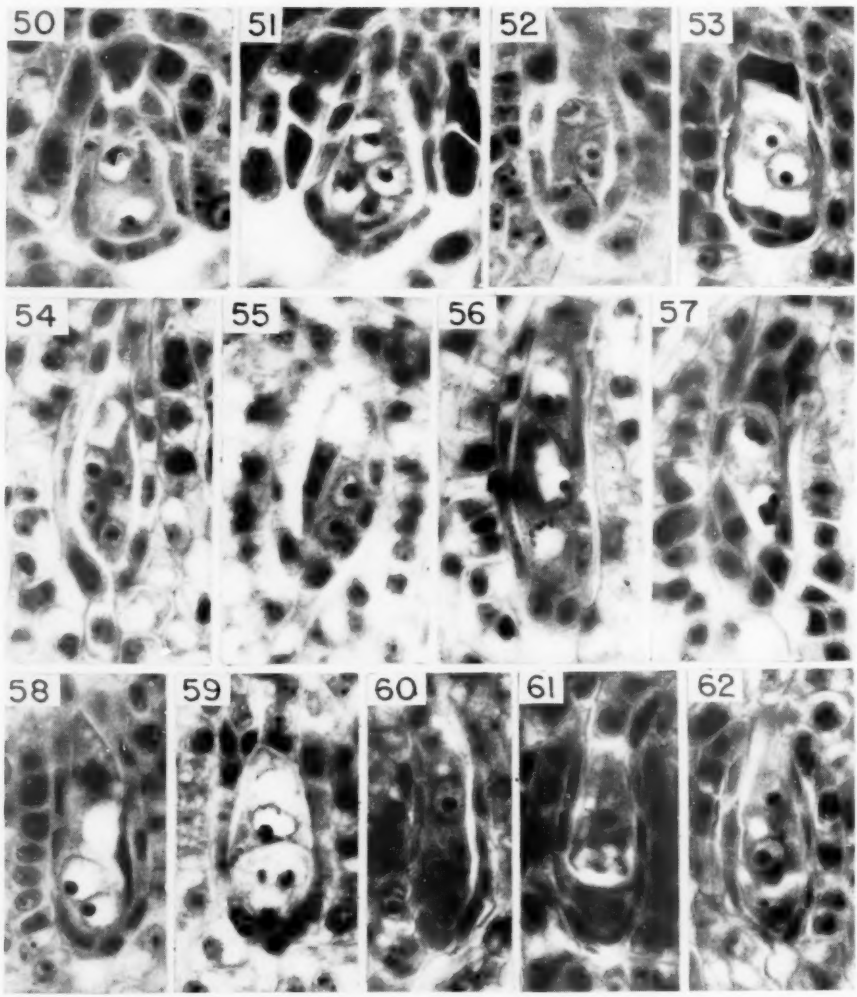
CASSIOPE MERTENSIANA.—The mature ovule is as described under *C. lycopodioides* (fig. 63, Cas. mert.). Because variation from the Polygonum Type occurs, as well as considerable variation in details of development among ovules in different flowers and even among ovules in the same flower (figs. 36–62), this species is of particular interest.

Cell division rarely, if ever, follows the first meiotic division, so that the dyad consists of a single cell containing two nuclei. The second meiotic division is usually followed by the formation of one,



FIGS. 36–49.—*Cassiope mertensiana*. Megasporogenesis and development of megagametophyte. Fig. 48 is of a stage not quite mature; it still shows disintegrating antipodals.

or rarely a second, wall. The wall generally is diagonal and separates two micropylar nuclei from two chalazal ones (fig. 40). Occasionally the wall is almost perpendicular with two nuclei on either side of it (fig. 54), or it is arc-shaped, separating two central but laterally located nuclei from the other two which occur terminally (fig. 52). The single wall may separate three micro-



FIGS. 50–62.—*Cassiope mertensiana*. Variations in megasporogenesis and megagametophyte development. Figs. 50, 51, precocious dyad and tetrad. Figs. 52, 53, arc-shaped wall in tetrad and functioning of central cell. Figs. 54, 55, almost perpendicular wall in tetrad and disintegration of one cell. Figs. 56, 57, two walls in tetrad with central binucleate cell becoming functional. Fig. 58, micropylar cell of usual tetrad going on. Figs. 59–62, tetrad to two-nucleate stage of Polygonum-Type development.

pylar nuclei from one chalazal one (fig. 59), or rarely the reverse. In one bud tetrads were found with three cells, the micropylar and chalazal with one nucleus, the central one binucleate (fig. 56).

In the few ovules in which three nuclei occur together micropylarly, they disintegrate, the chalazal cell with its single nucleus remaining functional (figs. 60–62). This gives rise in usual fashion to the later stages of the gametophyte, and hence development is essentially of the Polygonum Type. However, in the more frequently occurring condition of the tetrad with two binucleate cells, one cell disintegrates and the other cell, with its two spore-nuclei, functions directly as the two-nucleate gametophyte (figs. 42, 43), so that development is essentially of the Allium Type (**21**). When the wall is diagonal, usually the chalazal pair goes on (fig. 42), but occasionally the micropylar pair does (fig. 58); when the wall is arc-shaped, frequently the long, dumbbell-shaped cell with terminal nuclei functions, but occasionally the lateral one forms the gametophyte (fig. 53); when the wall is perpendicular, either cell may function (fig. 55). Rarely both two-nucleate cells of the tetrad will go on at least to the four-nucleate stage before one disintegrates.

Division in the four-nucleate stage is generally as expected, but occasionally during the later four-nucleate condition the chalazal nucleus becomes separated from the others and undergoes no further division. The three other nuclei divide normally, resulting in a total of seven nuclei. There may thus be only two antipodal cells; whether two or three, the antipodals disintegrate very early, so that no traces remain in most mature gametophytes (fig. 49). The synergids are similar to those of *C. lycopodioides*, and the polar nuclei rarely fuse.

Considerable variation occurs from one flower to another in the degree of development of the ovule between the megaspore mother cell and the two- and four-nucleate stages of the gametophyte. This variability, along with the occurrence of both Allium and Polygonum Types of development within a single ovary, led to the sectioning of more than one hundred buds, particularly from two collections of Alaskan[2] material which were made 2 weeks apart. In the earlier collection and in a few buds of the later one, an occasional dyad or tetrad was found along with many megaspore mother cells. In these the integument had reached only about the level of the tip of the nucellus, and the latter had not yet started to disintegrate (figs. 50, 51). Most of these tetrads showed no wall, but it may have been present in more of them because, as the wall is usually diagonal, it is not readily visible if it occurs in the plane of the section. The nuclei were similar in appearance to those of the megaspore mother cell—clear, with an irregular area of chromatin material—though they were smaller in size. In the later collection only occasional megaspore mother cells were found, and these appeared to show prophase nuclei (fig. 38). Several ovaries containing ovules with one or the other of the meiotic divisions were found (fig. 39); no such divisions were seen in the earlier material. Most of the ovules showed tetrads or some early stage in gametophyte development, and in most of them the nuclei were more normally granular in appearance. In these ovules the degree of development of the integument varied from ovules in which it had elongated

[2] Collections made by members of the Juneau Botanical Club.

beyond the nucellus but had not completely closed to form the micropyle to those in which development had reached essentially the stage found accompanying the mature gametophyte (figs. 38–41).

The nucellus remains intact through the tetrad stage, and its cells are still distinct, particularly at the micropylar end, in some ovules which have arrived precociously at the four-nucleate stage. In most ovules, however, the nucellus disintegrates during the two-nucleate stage and there is considerable enlargement of the gametophyte. Normally vacuoles do not occur in megaspore mother cell, dyad, or tetrad. They appear only with the beginning of disintegration of the micropylar spores and are characteristic of the gametophyte from functional spore or two-nucleate stage on. Occasionally, however, in elongated tetrads in which the nuclei occur micropylarly, vacuoles appear chalazally, and in one bud vacuoles were present even in the megaspore mother cell (fig. 38). Their appearance in the tetrad may be an indication of the initiation of gametophytic development which appears earlier than the disintegration of nonfunctional spores.

CASSIOPE TETRAGONA.—The mature ovule is similar to that described earlier under *C. lycopodioides* (fig. 63, Cas. tet.). Developmental stages are missing in the material at hand except for a few four-nucleate gametophytes. Material that otherwise might have shown stages was so poorly fixed that details could not be made out. The mature gametophyte appears very similar to that of *C. mertensiana* and *C. lycopodioides*.

CHAMAEDAPHNE CALYCULATA.—The mature ovule is intermediate in size, essentially campylotropous, the integument approximately five cell layers in thickness, and the endothelium usually well differentiated (fig. 63, Cham.). Almost complete developmental material is available (figs. 19–35); only the archesporial cell and its change to megaspore mother cell are missing. Again the rate of development is known for southern Michigan, where M. E. BRITTON made weekly collections from a single bog. In material collected in late October all stages from the megaspore mother cell to an early two-nucleate condition of the gametophyte were found, the majority of ovules showing some stage in the disintegration of the three micropylar spores. Rarely the spore next to the chalazal one becomes functional. The ovules apparently overwinter in the functional megaspore stage or in the early two-nucleate condition of the gametophyte because both are found in ovules collected in the middle of March. During the latter part of March the major enlargement of the gametophyte occurs while it is still two-nucleate (figs. 26–28). By the first week in April the four-nucleate condition is found, and the following week this divides to form the early eight-nucleate stage. During maturation of the gametophyte further enlargement occurs, particularly an expansion of the micropylar portion, which becomes quite large. The antipodals, consisting of two cells, are large, the chalazal cell with a single nucleus and the inner one with two. The polar nuclei usually do not fuse. They occupy a position close to the egg rather than at the constricted area between the egg apparatus and the antipodals (fig. 35). Disintegration of the nucellus occurs at the same time as the disintegration of the micropylar spores. It begins at the chalazal end and progresses micropylarly. Remains of the cells are visible during the functional megaspore and early two-nucleate stages, but essentially all traces have disappeared subsequent to that.

ENKIANTHUS CERNUUS.—The mature

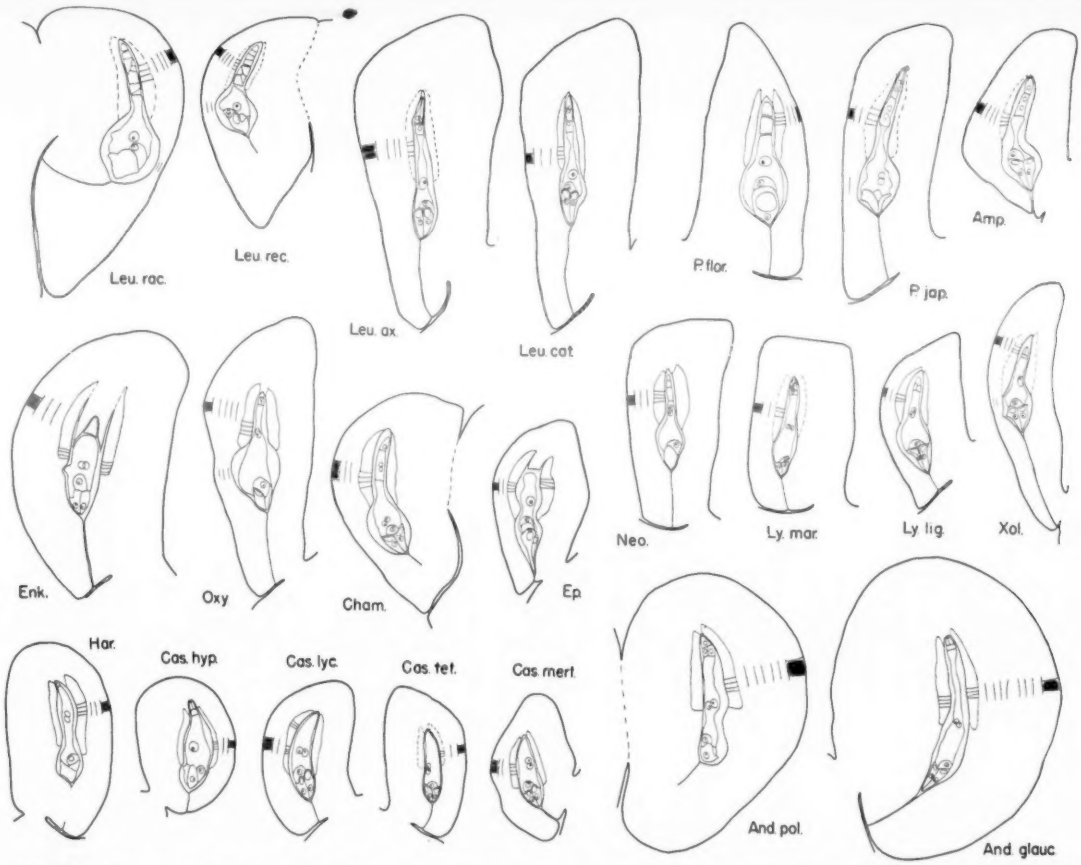


FIG. 63.—Diagrams of mature ovules of all species of Andromedeae studied, drawn at uniform magnification. Leu. rac., *Leucothoe racemosa;* Leu. rec., *Leucothoe recurva;* Leu. ax., *Leucothoe axillaris;* Leu. cat., *Leucothoe catesbaei;* P. flor., *Pieris floribunda;* P. jap., *Pieris japonica;* Amp., *Ampelothamnus phyllireifolius;* Enk., *Enkianthus cernuus;* Oxy., *Oxydendrum arboreum;* Cham., *Chamaedaphne calyculata;* Ep., *Epigaea repens;* Neo., *Neopieris nitida;* Ly. mar., *Lyonia mariana;* Ly. lig., *Lyonia ligustrina;* Xol., *Xolisma fruticosa;* Har., *Harrimanella stelleriana;* Cas. hyp., *Cassiope hypnoides;* Cas. lyc., *Cassiope lycopodioides;* Cas. tet., *Cassiope tetragona;* Cas. mert., *Cassiope mertensiana;* And. pol., *Andromeda polifolia;* And. glauc., *Andromeda glaucophylla.*

ovule is fairly large, essentially anatropous, with the endothelium well developed and extending chalazally beyond the end of the actual gametophyte (fig. 63, Enk.). The mature gametophyte area gives the general appearance of being elongated, but the gametophyte itself is actually quite short. The appearance results from a basal region of nucellar tissue which forms a sort of "pedestal" for the gametophyte (fig. 16). The mature gametophyte shows no enlargement of the micropylar portion but does have two peculiar earlike projections just posterior to the egg apparatus and anterior to the endothelium. The polar nuclei do not fuse, and the antipodals disintegrate, at least partially, prior to fertilization.

Surprisingly, two open flowers, comparable with ones showing mature gametophytes, contained one to two or three ovules in almost every stage of development. The only stages missing were those prior to the megaspore mother cell, of which only one was found, and the dyad. All stages are normal. The gametophyte tends to be rather broad throughout its development. Three antipodal cells are organized but begin disintegration early, so that their number is not evident in mature ovules. The "ears" found in the mature gametophyte are a late development, not occurring in younger eight-nucleate material. The endothelium differentiates very early, and the nucellus disintegrates more slowly than in most other species. Disintegration begins at about the time of division of the functional spore, and some nucellar cells may still be recognizable in four-nucleate stages.

EPIGAEA REPENS.—The mature ovule is rather small, slightly campylotropous, the integument approximately four cell layers in thickness, and the endothelium well differentiated (fig. 63, Ep.).

The stages between the archesporial cell and the disintegration of the spores are missing in the developmental material that has been observed. The fact that three disintegrating cells have been found distal to a single enlarged cell in several ovules would indicate that these stages must be essentially normal. Occasionally either a spore other than the chalazal one is found to be functional or two spores have been observed to enlarge simultaneously. The nucellar cells disintegrate along with the nonfunctional spores, and divisions to form two, four, and eight nuclei follow fairly closely without much enlargement of the gametophyte. The major enlargement occurs between the early eight-nucleate stage and the mature gametophyte. Older gametophytes sometimes appear to be "supported" by a "pedestal" similar to that in *Enkianthus cernuus*, though a much shorter one. Younger eight-nucleate material indicates, however, that this is usually made up of the antipodals, which soon after their formation frequently become highly vacuolate and appear distinct from the rest of the gametophyte, which is highly protoplasmic. The highly protoplasmic condition becomes evident in late four-nucleate material when the central vacuole essentially disappears. The antipodals later completely disintegrate. There is a constricted region just posterior to the egg apparatus (the result of the elongation of the endothelial cells closest to the micropyle) back of which the secondary nucleus usually occurs. STEVENS (**27**) stated that the polar nuclei do not fuse until fertilization, at which time there is a triple fusion. In my material a single nucleus, representing the fused polars, was found in many gametophytes prior to fertilization. Frequently several small, sharp-pointed "ears" project laterally at the

level of the egg. These appear slightly earlier in development than in *Enkianthus*. The synergids are extremely elongated and extend from near the region of the constriction almost to the opening of the micropyle. The egg lies near the constricted area: in it or just anterior or posterior to it (fig. 18).

HARRIMANELLA STELLERIANA.—The mature ovule is similar to that of *Cassiope*, though slightly larger and with approximately five cell layers in the integument (fig. 63, Har.). Developmental stages are not available for this species at the present time. The mature gametophyte may show some remains of antipodals. The polar nuclei normally are not fused.

LEUCOTHOE AXILLARIS.—The mature ovule is quite large, anatropous, the integument approximately six cell layers in thickness and the endothelium often weakly developed (fig. 63, Leu. ax.). From a single collection of buds and flowers, complete development has been followed. It corresponds to the usual pattern. Considerable enlargement occurs during the two-nucleate stage, but maximum enlargement occurs during the final maturation. The mature gametophyte is elongated with the micropylar end only slightly enlarged. The polar nuclei usually fuse. The antipodal cells are fairly large and start to disintegrate just about the time the gametophyte is mature. The three cells usually show a T-arrangement with the two cells at the chalazal end. The nucellus disintegrates during the tetrad stage.

LEUCOTHOE CATESBAEI.—The mature ovule is similar to that of *L. axillaris*, though the integument may show only five layers of cells (fig. 63, Leu. cat.). Fairly complete development is also available for this species, the stages missing being between the two-nucleate and mature gametophyte. The mature gametophyte is also similar to that of *L. axillaris* (fig. 15), though usually there are only two antipodal cells, the chalazal one being two-nucleated. Development apparently corresponds throughout in the two species.

LEUCOTHOE RACEMOSA.—The mature ovule is fairly large, essentially campylotropous, the micropyle curved back close to the region of attachment. The integument is about six cell layers in thickness over the chalazal end of the gametophyte but much less over the micropylar region, where it is crushed to three or less (rarely the gametophyte may actually break through in this area), and the endothelium is somewhat weakly developed (fig. 63, Leu. rac.). Developmental material is not available for this species at present. The mature gametophyte has two peculiarities—the extreme enlargement of the micropylar portion and the multiplication of the antipodal cells (fig. 17). The latter may vary from about six to fifteen in number and almost fill the narrow portion of the gametophyte which is surrounded by the endothelium. The polar nuclei fuse prior to fertilization, and the resulting secondary nucleus is located near the egg.

LEUCOTHOE RECURVA.—The mature ovule is similar to that of *L. racemosa* except that it is smaller (no reduction in number of cell layers, however) (fig. 63, Leu. rec.). The mature gametophyte is also similar, though the antipodals tend to be somewhat fewer in number. They are derived by multiplication of the three original cells. Essentially complete developmental material is available for this species. The enlargement of the gametophyte and the beginning of the expansion of the micropylar end occur in the two-nucleate stage. The nucellus begins disintegration during the tetrad stage. One

ovule is fairly large, essentially anatropous, with the endothelium well developed and extending chalazally beyond the end of the actual gametophyte (fig. 63, Enk.). The mature gametophyte area gives the general appearance of being elongated, but the gametophyte itself is actually quite short. The appearance results from a basal region of nucellar tissue which forms a sort of "pedestal" for the gametophyte (fig. 16). The mature gametophyte shows no enlargement of the micropylar portion but does have two peculiar earlike projections just posterior to the egg apparatus and anterior to the endothelium. The polar nuclei do not fuse, and the antipodals disintegrate, at least partially, prior to fertilization.

Surprisingly, two open flowers, comparable with ones showing mature gametophytes, contained one to two or three ovules in almost every stage of development. The only stages missing were those prior to the megaspore mother cell, of which only one was found, and the dyad. All stages are normal. The gametophyte tends to be rather broad throughout its development. Three antipodal cells are organized but begin disintegration early, so that their number is not evident in mature ovules. The "ears" found in the mature gametophyte are a late development, not occurring in younger eight-nucleate material. The endothelium differentiates very early, and the nucellus disintegrates more slowly than in most other species. Disintegration begins at about the time of division of the functional spore, and some nucellar cells may still be recognizable in four-nucleate stages.

EPIGAEA REPENS.—The mature ovule is rather small, slightly campylotropous, the integument approximately four cell layers in thickness, and the endothelium well differentiated (fig. 63, Ep.).

The stages between the archesporial cell and the disintegration of the spores are missing in the developmental material that has been observed. The fact that three disintegrating cells have been found distal to a single enlarged cell in several ovules would indicate that these stages must be essentially normal. Occasionally either a spore other than the chalazal one is found to be functional or two spores have been observed to enlarge simultaneously. The nucellar cells disintegrate along with the nonfunctional spores, and divisions to form two, four, and eight nuclei follow fairly closely without much enlargement of the gametophyte. The major enlargement occurs between the early eight-nucleate stage and the mature gametophyte. Older gametophytes sometimes appear to be "supported" by a "pedestal" similar to that in *Enkianthus cernuus*, though a much shorter one. Younger eight-nucleate material indicates, however, that this is usually made up of the antipodals, which soon after their formation frequently become highly vacuolate and appear distinct from the rest of the gametophyte, which is highly protoplasmic. The highly protoplasmic condition becomes evident in late four-nucleate material when the central vacuole essentially disappears. The antipodals later completely disintegrate. There is a constricted region just posterior to the egg apparatus (the result of the elongation of the endothelial cells closest to the micropyle) back of which the secondary nucleus usually occurs. STEVENS (27) stated that the polar nuclei do not fuse until fertilization, at which time there is a triple fusion. In my material a single nucleus, representing the fused polars, was found in many gametophytes prior to fertilization. Frequently several small, sharp-pointed "ears" project laterally at the

level of the egg. These appear slightly earlier in development than in *Enkianthus*. The synergids are extremely elongated and extend from near the region of the constriction almost to the opening of the micropyle. The egg lies near the constricted area: in it or just anterior or posterior to it (fig. 18).

HARRIMANELLA STELLERIANA.—The mature ovule is similar to that of *Cassiope*, though slightly larger and with approximately five cell layers in the integument (fig. 63, Har.). Developmental stages are not available for this species at the present time. The mature gametophyte may show some remains of antipodals. The polar nuclei normally are not fused.

LEUCOTHOE AXILLARIS.—The mature ovule is quite large, anatropous, the integument approximately six cell layers in thickness and the endothelium often weakly developed (fig. 63, Leu. ax.). From a single collection of buds and flowers, complete development has been followed. It corresponds to the usual pattern. Considerable enlargement occurs during the two-nucleate stage, but maximum enlargement occurs during the final maturation. The mature gametophyte is elongated with the micropylar end only slightly enlarged. The polar nuclei usually fuse. The antipodal cells are fairly large and start to disintegrate just about the time the gametophyte is mature. The three cells usually show a T-arrangement with the two cells at the chalazal end. The nucellus disintegrates during the tetrad stage.

LEUCOTHOE CATESBAEI.—The mature ovule is similar to that of *L. axillaris*, though the integument may show only five layers of cells (fig. 63, Leu. cat.). Fairly complete development is also available for this species, the stages missing being between the two-nucleate and mature gametophyte. The mature gametophyte is also similar to that of *L. axillaris* (fig. 15), though usually there are only two antipodal cells, the chalazal one being two-nucleated. Development apparently corresponds throughout in the two species.

LEUCOTHOE RACEMOSA.—The mature ovule is fairly large, essentially campylotropous, the micropyle curved back close to the region of attachment. The integument is about six cell layers in thickness over the chalazal end of the gametophyte but much less over the micropylar region, where it is crushed to three or less (rarely the gametophyte may actually break through in this area), and the endothelium is somewhat weakly developed (fig. 63, Leu. rac.). Developmental material is not available for this species at present. The mature gametophyte has two peculiarities—the extreme enlargement of the micropylar portion and the multiplication of the antipodal cells (fig. 17). The latter may vary from about six to fifteen in number and almost fill the narrow portion of the gametophyte which is surrounded by the endothelium. The polar nuclei fuse prior to fertilization, and the resulting secondary nucleus is located near the egg.

LEUCOTHOE RECURVA.—The mature ovule is similar to that of *L. racemosa* except that it is smaller (no reduction in number of cell layers, however) (fig. 63, Leu. rec.). The mature gametophyte is also similar, though the antipodals tend to be somewhat fewer in number. They are derived by multiplication of the three original cells. Essentially complete developmental material is available for this species. The enlargement of the gametophyte and the beginning of the expansion of the micropylar end occur in the two-nucleate stage. The nucellus begins disintegration during the tetrad stage. One

variation that occasionally occurs is that two spores may start to develop. Development is then usually considerably delayed. One may gain ascendancy over the other, and it completes development. In one instance a gametophyte was observed in which the major portion had penetrated through the micropyle and was located between the ovule and the placenta. The chalazal portion remained within the ovule.

LYONIA LIGUSTRINA.—The mature ovule is relatively small, essentially anatropous, the integument approximately four cell layers in thickness, and the endothelium rather well developed (fig. 63, Ly. lig.). The mature gametophyte is somewhat variable in shape but is usually somewhat elongated, with the micropylar region moderately expanded. Occasionally it may be rather short and broad throughout. Essentially complete development is available for this species, and it follows the usual pattern. Sometimes the micropylar dyad cell disintegrates without the nucleus dividing. When this occurs, the chalazal cell completes meiosis and the chalazal one of the resulting two spores gives rise to the gametophyte. Even when it divides, the micropylar cell disintegrates earlier than the chalazal. The synergids of the mature gametophyte may occasionally elongate micropylarly for a considerable distance. The polars do not fuse prior to fertilization. The antipodals are small, variously arranged, and start to disintegrate early. The nucellus disintegrates along with the micropylar spores, but its remains are still distinct in the two-nucleate stage. Gradual enlargement occurs throughout development, but a large amount occurs between the early eight-nucleate condition and complete maturity of the gametophyte.

LYONIA MARIANA.—The mature ovule is rather small, anatropous, and in general rather similar to that of *L. ligustrina* (fig. 63, Ly. mar.). Developmental stages are also similar throughout except that a normal tetrad is formed. The mature gametophyte is also similar, though here the antipodals are typically arranged in a T with the two cells at the chalazal end. The synergids normally do not elongate to any extent. Disintegration of the nucellus again accompanies disintegration of the spores, lasting into the two-nucleate stage, and enlargement occurs primarily between the early eight-nucleate and mature gametophyte stages, largely as a result of a forward projection into the micropylar region.

NEOPIERIS NITIDA.—The mature ovule is slightly larger than that of *Lyonia mariana*, the integument of about five cells, but is otherwise similar (fig. 63, Neo.). Early developmental (up to early two-nucleate) and the mature gametophyte stages are available for this species. A single archesporial cell is present. A tetrad is formed, and of this the three micropylar spores disintegrate. The mature gametophyte is essentially similar to that in *Lyonia mariana*, but the antipodals are linearly arranged. Disintegration of the nucellus begins with the disintegration of the spores, but the micropylar cells are still distinct in the early two-nucleate condition.

OXYDENDRUM ARBOREUM.—The mature ovule is medium in size, anatropous, with the integument about seven cells in thickness around the chalazal portion of the gametophyte but crushed to about four around the expanded micropylar portion, and the endothelium is fairly well differentiated (fig. 63, Oxy.). Complete development has been found except for a young tetrad. Apparently the micropylar dyad cell may occasionally fail to divide, as in some ovules only two dis-

integrating cells occur distal to the functional spore. The two-nucleate gametophyte elongates micropylarly, so that frequently its distal nucleus lies beside the disintegrating spores. Gradual elongation occurs throughout development, and the seven cells of the mature gametophyte are organized before an expansion occurs at the micropylar end. The antipodals are small from the beginning (even the chalazal two nuclei of the four-nucleate stage may frequently be small and be accompanied by little cytoplasm), and they disintegrate early. Great micropylar enlargement occurs during maturation of the gametophyte, and the egg apparatus, organized before this enlargement, appears small with respect to the area it occupies. The polar nuclei remain unfused throughout this expansion but fuse just prior to fertilization.

PIERIS FLORIBUNDA.—The mature ovule is medium in size, essentially anatropous, the integument about five cell layers in thickness, and the endothelial layer fairly well differentiated (fig. 63, P. flor.). Complete development has been followed for this species and corresponds to the usual pattern. Rarely the megaspore next to the chalazal one gives rise to the gametophyte. Considerable enlargement occurs in the two- and four-nucleate stages, so that little occurs in the final maturation of the gametophyte. Disintegration of the nucellus begins chalazally as the functional spore begins to enlarge and the other cells to disintegrate. Remains, particularly of the micropylar nucellar cells, are found outside the earlier two-nucleate gametophytes. The mature gametophyte is rather large, broader than in many other species, with the micropylar end expanded. The polar nuclei fuse prior to fertilization, and the secondary nucleus is located approximately at the point of constriction. The antipodal cells are quite large, variously arranged, but frequently in a T with the two at the chalazal end. Their disintegration begins at about the time of fertilization.

PIERIS JAPONICA.—The mature ovule is similar in size to that of *P. floribunda*, though the integument may show only four cell layers, with the endothelial layer not clearly differentiated (fig. 63, P. jap.). Developmental material is not at present available for this species. The mature gametophyte is narrower at the chalazal end than in *P. floribunda*, with the micropylar portion considerably expanded. The egg apparatus is not so large, and the unfused polar nuclei are located near the egg. The antipodal cells are rather large and linearly arranged. Apparently the nucleus of the micropylar antipodal cell may divide so that this cell may be binucleate, and occasionally the cell also divides so that four cells are found. The antipodals begin to disintegrate at about the time the gametophyte attains maturity.

XOLISMA FRUTICOSA.—The mature ovules are relatively long but quite thin, so that the total size is only slightly greater than that of *Lyonia ligustrina*. The integument is about five cell layers in thickness at the chalazal end of the gametophyte, less where the micropylar portion is expanded. The endothelial layer is rather clearly differentiated (fig. 63, Xol.). Except for the archesporial cell, the developmental story is complete for this species and is similar to that in *Lyonia ligustrina* except that the micropylar dyad cell usually divides, giving rise to a normal tetrad. The mature gametophyte looks relatively short as compared with the length of the ovule, but it is no more so than the commoner shape in *L. ligustrina*. The mature gametophyte

is similar in most respects to that in the latter species.

Discussion

Many of the general characters described here for the Andromedeae correspond with those which have been described by others for various members of the Ericales: single integument, a single-layered evanescent nucellus, a more or less differentiated endothelium, and the Polygonum Type of development of the megagametophyte from a single archesporial cell, which functions directly as the megaspore mother cell. The ovule is usually reported to be anatropous but in certain species is considered to approach the campylotropous condition.

The single integument varies in thickness from two or three layers in the Pyroleae (**6, 15, 25**) and Monotropoideae (**6, 7, 8, 10–13, 18, 25**) to four to six in most other species, with thicker integuments reported for *Daboecia* in the Rhododendroideae (**14**), and *Styphelia* in the Epacridaceae (**4**); in neither of these, however, is the integument so heavy as that found in *Andromeda glaucophylla*. The outer layer of the integument usually appears to contain tannin-like material, as reported for the Andromedeae. The degree of differentiation of the endothelium varies from none in *Pholisma* (**9**) (Lennoaceae, questionably a family of the Ericales) to only slightly in many of the Monotropoideae (**6, 12, 13, 25**) and a few of the Pyroleae (**6, 15**) to very distinct in many of the Rhododendroideae (**6, 14, 25**) and in the species of Clethraceae (**19**), Arbuteae (**25**), Gaultherieae (**5, 25**), Ericoideae (**3, 25**), and Vaccinioideae (**25**) that have been reported. In the Andromedeae the weakest development of the endothelium is found in the very small ovules of the *Cassiope* group, and the greatest development in *Enkianthus* and *Epigaea*. In addition to the differentiation of an endothelium, certain species are reported as having additional cells of the integument at the chalazal, and occasionally at the micropylar, end become highly protoplasmic. There is only a slight development of this type at the chalazal end of certain species of the Andromedeae (*Oxydendrum*), but it is reported to be characteristic of certain species of *Clethra* (**19**) and *Vaccinium* (**25**).

Cassiope mertensiana, described in the present paper, is the only species which has been reported to have a gametophyte developing according to any pattern other than Polygonum Type. In this species, although Polygonum Type may occasionally be found, the usual pattern of development corresponds to a modified Allium Type. The only other report of a species of *Cassiope* which gives any indication of development is SAMUELSSON's brief description of *C. hypnoides* (**26**). This description is mostly of seed development, but the implication is that gametophyte development of all species studied is normal (Polygonum Type). Attempts are being made to obtain additional material of the other species of *Cassiope* and of *Harrimanella* to see whether they agree with *C. mertensiana* in developmental pattern.

The modifications which occur in the usual Allium Type are the lack of cross-wall in the dyad and the division of both nuclei of the dyad with the single wall forming only in the tetrad stage. In the more typical Allium Type the dyad nuclei are separated by a wall, the micropylar cell disintegrates, and the nucleus of only the chalazal cell divides; the two spore nuclei are not separated by a wall and function directly as the two-nucleate gametophyte.

Some variations have been reported with respect to tetrad condition during

normal development. Typically a linear tetrad is formed with the chalazal spore functioning. A T-tetrad has been reported for *Pholisma* (**9**) and *Pleuricospora* (**6**). A failure of the micropylar dyad cell to divide, thus giving rise to a linear row of three cells, as seen in *Lyonia ligustrina* and *Xolisma fruticosa*, has been described for *Sarcodes* by OLIVER (**22**) and COPELAND (**6**), but DOYEL and GOSS (**18**) described a typical tetrad. A peculiar condition is that reported by BROUGH (**4**) for *Styphelia longifolia*. In this species reduction divisions are not followed immediately by wall formation. Membranes are later formed, however, cutting off three chalazal cells and leaving the micropylar one to function in the formation of the gametophyte. The three chalazal spores do not disintegrate, but enlarge and are considered to be haustorial.

Just as in the Andromedeae, the development and persistence of the antipodals vary considerably in other members of the Ericales. They are large, though they may disintegrate early, in several species of *Erica* (**25**), in *Calluna* (**3, 25**), some species of *Gaultheria* (**5, 25**), *Clethra* (**19**), *Bruckenthalia* (**25**), etc., while in others, such as *Newberrya* (**7**), *Pholisma* (**9**), *Kalmia* (**14, 25**), *Menziesia* (**14, 25**), and *Styphelia* (**4**), they are very small or have essentially completely disappeared before fertilization. No species are described as having multiplied antipodal tissue, such as occurs in *Leucothoe racemosa* and *L. recurva*. The condition of the polar nuclei, whether fused or unfused, at the time of fertilization also varies in other groups besides the Andromedeae.

In all forms that have been reported the gametophyte projects anteriorly following the disintegration of the nucellus, so that the egg apparatus occurs micropylarly to the endothelial layer and may bulge considerably. The position of the polar nuclei (or secondary nucleus) is variable with respect to the egg, occurring close to the egg in some species and back of the constriction caused by the beginning of the endothelial layer in others. The extremely elongated condition of the synergids found in *Epigaea* appears to be characteristic of many members of the Rhododendroideae (**14**). No report of the "pedestal" characteristic of *Enkianthus* and the "ears" in *Enkianthus* and *Epigaea* has been found for other species.

The above survey of ovule and gametophyte characteristics that are known indicates that there is no character or combination of characters which separate the Andromedeae as now constituted from other tribes of the Ericaceae or families of the Ericales. The Andromedeae essentially agree with all other reported members of the Ericales with respect to the first three characters listed as being of taxonomic significance in the introduction to this paper. In general pattern *Cassiope mertensiana* is the only species which is known to vary with respect to the fourth, though there is variation in a few details (not specific to any one group). There is some variation in all groups with respect to the fifth character. This variation, as it occurs within the Andromedeae, may be useful in suggesting narrow ranges of affinity within the tribe. Figure 63 contains diagrams of the mature ovules of all species studied, drawn at a uniform magnification. These indicate the relative sizes of the ovules, the number of layers in the integument, the approximate degree of development of the endothelium, and the various characteristics of the mature megagametophyte. The diagrams are arranged as nearly as possible to conform to the affinities of the species tentatively suggest-

ed in the earlier paper (**23**). It is interesting and encouraging to note the relatively good correspondence of variation in ovular characteristics with the variation in vascular anatomy and organography of the flower.

Leucothoe racemosa and *L. recurva* are similar to each other and different from all others in the possession of multiplied antipodals. In addition, the ovules in these species are campylotropous, the micropylar end of the gametophyte is greatly enlarged, and the polars fuse prior to fertilization. Campylotropous ovules are found in *Chamaedaphne* and *Andromeda*, but in neither of these are the polars found fused, and in the latter the micropylar end of the gametophyte is only slightly enlarged. The other two species of *Leucothoe*, *L. axillaris* and *L. catesbaei*, agree with the first two in having quite large ovules and fused polars, but the ovules are anatropous, the micropylar end of the gametophyte only slightly enlarged, and the antipodals not multiplied, though they are fairly large.

The shape and, to a slight extent, the size of the essentially anatropous ovules in the *Pieris* group vary with position on the placenta, lateral ones resembling those shown for *P. floribunda* and *Ampelothamnus*, the central ones similar to that shown for *P. japonica*. The gametophytes agree in being enlarged micropylarly and in having relatively large antipodals.

In the *Lyonia* group there is considerable similarity in the gametophytes of *L. ligustrina* and *Xolisma fruticosa*, though the ovule of the latter is considerably longer than that of the former. *Neopieris nitida* and *L. mariana* also show certain similarities, particularly in general ovular shape. All four have relatively small antipodals, and in this, in their general size, and in the moderate micropylar swelling of the gametophyte are more similar to one another than to the two previous groups.

The four species originally considered to be fairly distinct from the others (*Epigaea*, *Enkianthus*, *Oxydendrum*, *Chamaedaphne*) also show peculiar combinations of ovular characteristics, particularly *Epigaea repens* and *Enkianthus cernuus*.

Andromeda, as on the basis of vascular anatomy and organography, is quite distinct from the other species. *A. polifolia* has a slightly smaller ovule and gametophyte than *A. glaucophylla*, but other features of the two are quite similar.

The ovules of the *Cassiope* group also correspond to the earlier grouping: *C. mertensiana*, *C. lycopodioides*, and *C. tetragona* are essentially indistinguishable from one another; *Harrimanella stelleriana* is most distinct with *C. hypnoides* somewhat intermediate. The mature ovule material of the last two species was not so good as could be wished. Better material is needed to clarify the situation here.

As far as any general phylogenetic tendencies are concerned, evidence from the megagametophyte corroborates that from the vascular anatomy of the flower. On the basis of the latter, *Leucothoe racemosa* and *L. recurva* were considered most primitive in the Andromedeae because of the three-trace, three-gap vascular supply to each sepal. According to BATTAGLIA (**2**) "Polyantipody, in the phylogeny of the angiosperms, should . . . be an indication of primitiveness. . . . Thus this last situation [Normal (Polygonum) Type plus polyantipody] must be regarded as the most primitive embryological behaviour in angiosperms." This is the embryological condition char-

acteristic of both these species of *Leucothoe*. On the other hand, a change to a bisporic or tetrasporic condition and an early disappearance of the antipodals are considered to be derived (**20**). Both are found in *Cassiope* (at least *C. mertensiana* has been shown to be bisporic). This is combined with a vascular supply of a single trace to each sepal and considerable nondivergence of traces—an advanced vascular condition. *Andromeda*, which also shows an advanced vascular condition, has a much heavier integument than any others of the Andromedeae, but the antipodals are considerably reduced. The increased size of the ovule is probably associated, at least in part, with the relatively small number of ovules per carpel as compared with the other species of the tribe.

There is no good evidence from this study of the megagametophyte for allying *Enkianthus* and *Epigaea*, which do have a few features in common, with *Cassiope* in a new tribe, Cassiopeae, as was done by Cox (**16**) on the basis of the anatomy of the secondary wood.

When the necessary material is at hand, stamen development, microsporogenesis, endosperm development, and embryogeny will be investigated to determine what evidence they can contribute toward establishing relationships within the Andromedeae and between the Andromedeae and other groups of the Ericales.

Summary

1. The mature ovules of twenty-two species of the Andromedeae and fairly complete development of the megagametophyte of fifteen of these are described.

2. Certain features are characteristic of the ovules of all species: single integument, single-layered evanescent nucellus, archesporium of a single cell which gives rise directly to the megaspore mother cell, the cells of the outer layer of the integument containing a tannin-like substance, the inner layer of the integument more or less differentiated as an endothelium in the region of the nucellus, the ovules anatropous to campylotropous, and the penetration of the micropyle by the megagametophyte after the disintegration of the nucellus.

3. With the exception of one species, a single developmental type is found for all species studied in detail: Polygonum Type. The tetrad is linear, and the three micropylar spores normally disintegrate, leaving the chalazal one to function.

4. Development in *Cassiope mertensiana* usually corresponds to a modified Allium Type. No walls are formed during meiosis. Usually a single wall develops during the tetrad stage, separating two nuclei from two. One two-nucleate cell disintegrates, and the other functions directly as the two-nucleate gametophyte. Wall formation and disintegration in the tetrad are variable, and an essentially Polygonum Type development is occasionally found.

5. Variation occurs in ovule size, number of layers in the integument, size and fate of the antipodals, shape of the synergids, time of fusion of the polar nuclei, degree of bulging of the micropylar end of the mature gametophyte, and extent of bending of the ovule and gametophyte toward the campylotropous condition.

6. Ovule and megagametophyte characteristics in the Andromedeae correspond to those of the others of the Ericales that have been described, except for the Allium-Type development in *Cassiope mertensiana*.

7. Variations in ovule characteristics within the Andromedeae show consider-

able agreement with the affinities tentatively suggested in an earlier paper based on the vascular anatomy and organography of the flower.

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DEPARTMENT OF BOTANY
UNIVERSITY OF CHICAGO

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INTERRELATIONS OF SELECTED PLANT CHARACTERS IN KENTUCKY BLUEGRASS (POA PRATENSIS L.)[1]

E. L. NIELSEN AND D. C. SMITH[2]

Introduction

In the course of several years of nursery investigations with *Poa pratensis* L. at Madison, Wisconsin, spaced plants of numerous strains from many states and foreign countries have been grown. Many plant types have appeared, these representing various combinations of characters, fifteen of which have been observed in some detail. Since the species has long been recognized as being extremely polymorphic, considerable interest existed in interrelations of the numerous characters and their possible associations in prominent types or complexes. The present discussion is concerned with results of studies of character relationships and their possible use in type selection.

Review of literature

The European literature is replete with studies concerning the variations and relationships of characters in some species of the grasses. The majority of these studies have been based upon rather extensive collections, but statistical analyses of the data have not been made in many instances. Some workers have selected representative plants from grass populations and have presented detailed observations in some of the more pertinent literature.

DIX (**4**) investigated *Lolium italicum* A. Br., *L. perenne* L., *Arrhenatherum elatius* (L.) Presl., *Alopecurus pratensis* L., and *Avena* (*Trisetum*) *flavescens* L. The characteristics of one form of *L. perenne* and twenty-seven forms of *L. italicum* were presented in tabular form. HESSING (**6**), from a study of the leaf shape of *L. italicum* and one of its varieties (var. *westerwoldicum*) and *L. perenne*, concluded that the percentages in the different leaf-shape classes (V-shape, U-shape, flat, or intermediate) varied annually but that the progenies usually resembled the parent in the character studied. The relation of spike length to the number of spikelets was a highly variable character in *L. italicum*.

WITTE's (**14, 15**) extensive work with *Phleum pratense* L., *Dactylis glomerata* L., and other forage grasses included observation of a number of morphological and physiological characters. A tabular description was presented for fifteen strains of timothy and sixteen strains of orchard grass (**14**). Additional information extending these observations, together with parent-progeny relations, was included in a second series of papers (**15**). WITTE concluded the studies with a discussion of the important characters for practical utilization of timothy.

SELECHTNER's (**11**) studies concerning *Agrostis stolonifera* L. were based upon a minimum of a hundred and fifty observations each, on such characteristics as adventitious root formation, leaf innova-

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[2] Agronomist, Division of Forage Crops and Diseases, BPISAE, and Professor of Agronomy, University of Wisconsin.

tions, ligule length, rhizome features, the number, width, and length of leaves, culm formation, etc. Some of the types included in his collective species, *A. stolonifera* L., might usually be relegated to either *A. alba* L. or *A. vulgaris* With. There was a high frequency of intergrading forms.

WIELAND (**13**) found no relationship occurring in *Lolium perenne* between growth form and leafiness, culm formation, number and size of spikelets, and spike size and the number of florets. A significant negative relation was found between number of culms and leafiness, a relationship that also occurred in *Poa pratensis*. Extensive data are included, based upon fifty observations for each character, for fifteen types of Kentucky bluegrass and sixteen types of *L. perenne*.

CLARK (**3**) reported that in timothy populations the largest coefficient of variability occurred in the plant weights and the smallest in plant height. Low correlations were determined between such characters as earliness and plant height, earliness and weight, duration of flowering and plant height, and duration of flowering and weight. The study was conducted over a period of 3 years. Correlations between years were .38 and .55 for plant weight and .49 and .59 for plant height.

FISCHER (**5**), studying individual plants of meadow fescue, found the coefficients of variability in the descending order for magnitude in rust occurrence, culm diameter, leaf yield of aftermath growth, and green weight. He concluded that the correlations determined were of little value. Those regarded to be most significant were negative and included relations between length and number of culms, coarseness and number of culms, and length and coarseness of culms.

WALDRON (**12**) compared two strains of *Bromus inermis* Leyss. and found coefficients of variability in plant height approximating 23.5% for the Canadian stock and 19.8% for that from South Dakota. Interannual coefficients of variability in plant weight during the 3 years studied were from 20.5 to 35.1 for the Canadian and from 23.2 to 35.6 for the South Dakota strain. Correlation coefficients for plant height and plant weight during the 3 years were .33, .23, and .36 for the South Dakota strain, and .51, .35, and .40 for the Canadian strain.

JACQUES (**7**) concluded that the smaller, slow-growing, fine-leaved, and longer-lived strains of crested dogstail (*Cynosurus cristatus* L.) were at a disadvantage in competition with the stemmy, broad-leaved, early-maturing strains.

NIELSEN (**9**) reported highly significant correlations to occur in the plant height of *Panicum virgatum* L. in relation to panicle length, length of the lowermost panicle branch, length of the upper internode, length and width of the uppermost and fourth leaves, and the diameter of the first and fourth nodes. No relations were found between plant height and the length of rhizome internodes, number of rhizome nodes, and spikelet and caryopsis lengths. Panicle length was closely related to the length of its lowermost branch but not related to the length of the spikelet or caryopsis.

ROSSITER (**10**) reported that a highly significant relation occurred between seed weight and seed color in *Ehrharta calycina* Sm. The length of time between the exsertion of the panicles and flowering was greater in the early short types than for tall late types. Likewise, decumbent growing forms were significantly earlier in flowering and of greater perenniality than those of ascending habit.

There are few detailed, comparative data concerning specific characters of Kentucky bluegrass. The only extensive

study noted comparing several definite morphological features was described by BÄR (**1**). He observed 90 plants of different wild populations. The creeping habits of the different forms were compared on the basis of the number of clusters or rosettes from rhizomes with the average yield and the relative percentage of yield from the different types in the average yield group. He was unable to find a significant difference among 2040 plants of 32 different strain types for habit of spreading. In addition, fourteen correlations were determined between certain characters of the several types. Since the periodical in which these results were published is not generally available, the pertinent associations he determined are included as follows:

	r values
Leaf width to basal leaf yield	0.09±0.11
Culm length to leaf width	−.10± .12
Leaf width to relative stem weight	.60± .13
Leaf width to basal leaf yield	−.18± .13
Leaf length to basal leaf yield	.34± .10
Leaf length to leaf width	−.33± .09
Leaf length to leaf width to basal leaf weight	.10± .11
Leaf length to basal leaf yield	.06± .13
Leaf length to culm length	0.54±0.08

Based upon a study of herbarium specimens, BROWN (**2**) reported significant positive correlations between leaf width and the number of florets per spikelet and width of panicle and also between leaf width and the number of rhizomes. He concluded that variation was not random and that certain characters tended to remain together. Upon such a basis Kentucky bluegrass plants appeared to fall into two main subdivisions: (*a*) plants with narrow leaves (1.5–2.0 mm. broad) had small spikelets (about three florets), coarse, stiff, wiry roots, and few rhizomes and (*b*) plants with wider (3.5–5.0 mm. broad) and shorter leaves had larger spikelets (four to five florets) and numerous and longer rhizomes. He also suggested that this variation might be regulated by two distinct genomes being present in the respective types.

MYERS and GARBER (**8**) concluded from a bluegrass strain study that productivity was not always associated with aggressiveness. Certain strains of bunch- or tussock-type growth habit were sometimes more productive than their more prostrate and aggressive counterparts.

Methods of study

The data presented were compiled during the summers of 1941 and 1943. Approximately 1150 of the 3800 spaced plants grown in nurseries in 1941 were studied in detail. Among individual progenies a single plant of the predominant type and all aberrant plants of deviating morphological type were included. The data were classified and assembled for normal, or predominant (apomicts), and for aberrant (sexually reproduced) plants.

For present purposes there is little reason for separating the data for normal and aberrant plants as given in table 1 (group 1, 1941). No separation was made for normal and aberrant plants in the analysis of 1943 (group 2) data. Instead approximately 1050 plants were selected from nearly 15,000 grown in nurseries that year. The selected plants included a wide range of types.

The plants were classified for fifteen characters in 1941. Owing to inclement weather it was not possible to study certain of these characters in 1943. All traits considered were of interest in the estimation of possible pasture and turf value. Some, such as early vigor, suggest relative amounts of growth produced at the beginning of the growing season and are variable through the year. Others, as

growth habit and color, may remain relatively constant during the season. The features examined are listed. These were evaluated according to the scales indicated.

Plant height: measured in inches
Vigor: 1, vigorous, with intermediate grades to 5, very weak
Early and late habit: 1, prostrate; 2, near prostrate; 3, intermediate; 4, near tussock; 5, tussock
Leaf length: 1, long, with intermediate grades to 3, short
Leaf width: 1, wide (coarse), with intermediate grades to 5, narrow (fine)
Number of leaves: 1, numerous, with intermediate grades to 3, sparse
Aggressiveness: 1, aggressive, with intermediate grades to 3, nonaggressive (4, 5, very weak)
Size of culms: 1, fine, with intermediate grades to 3, coarse
Number of culms: 1, many, with intermediate grades to 3, few; 4, none
Foliage color: 1, light green; 2, medium green; 3, dark green; 4, light blue-green; 5, medium blue-green; 6, dark blue-green
Seed maturity: 1, early, with intermediate grades to 5, late
Leaf earliness: 1, early, with intermediate grades to 5, late
Panicle type: 1, open (shape pyramidal, branches perpendicular to axis, longer), with intermediate grades to 4, closed (panicle branches short, appressed to axis)

It is recognized that the types studied may not be representative of the entire range of variations of *P. pratensis*. Attention is also directed to the fact that certain types are encountered only rarely, if at all, in naturally occurring mass stands of the species in north-central United States. Examples of such types include very fine, dark-green plants often having purplish panicles, and others of extreme tussock-type growth, short yellow-green leaves, and panicles that barely extend beyond the leaves. Likewise, dark-green or strongly glaucous, coarse-textured plants are encountered only rarely in this region. All these types are included among the plants analyzed.

The data for the several characters were tabulated for years and according to normalcy or aberrancy in 1941. Normal types were those resembling the majority of plants of the progeny, owing primarily to apomixis. Aberrant plants were considered to be those not conforming to the major type, probably resulting from sexual reproduction. These were analyzed using the chi-square test for independence.

Results

The associations included in table 1 have been determined. The agreement between the comparisons for the 2 years is generally good. In several instances nonsignificant tendencies disagree in their directions. A significant difference in direction of association was found for the comparison of size of culms to the number of culms. Associations are generally negative (highly significant) for these characters in group 1*a* and for all plants in group 2, whereas it is significantly positive for the aberrant plants in group 1*b*. The only explanation for this appears to be that among the plants studied in group 1 there was a disproportionately large number of extreme tussock-type, fine-textured plants with relatively few fruiting culms. Among coarse-textured, aggressive types were frequent aberrants with numerous culms. It appears that the latter must have been sufficiently numerous to influence the direction of the association among the aberrants.

It appears unnecessary to review in detail the individual associations indicated in the table. Instead, certain implications and tendencies that may be of interest for the evaluation of turf and pasture

TABLE 1

INTERRELATIONS OF CHARACTERS IN POA PRATENSIS AS INDICATED BY *F* VALUES DERIVED FROM CHI-SQUARE TESTS FOR INDEPENDENCE

CHARACTER RELATIONS	GROUP 1				GROUP 2	
	a (normal)		*b* (aberrant)			
	No. plants	*P**	No. plants	*P**	No. plants	*P**
As plant height increases:						
early vigor increases	329	3	825	1	1018	1
late vigor decreases	326	1	776	1	1002	1
early habit becomes tussock	291	3	746	3	1030	1
late habit becomes tussock	214	1	594	1	.....	.....
leaf length increases	219	3	608	1	1032	1
leaf width decreases	244	3	695	3	1025	0
no. leaves increases	246	3	667	3	1028	3
aggressiveness increases	270	3	681	1	1038	1
size of culms decreases	207	1	606	1	1014	1
no. culms increases	231	1	668	1	1023	1
leaf earliness decreases	280	3	706	1	.....	.....
foliage color becomes light green	209	3	670	3	1014	1
seed maturity becomes later	204	2	638	1	1022	2
panicle type becomes closed	231	1	657	3	.....	.....
As early vigor decreases:						
late vigor decreases	334	1	661	1	920	1
early habit becomes tussock	298	1	493	1	1039	1
late habit becomes tussock	234	0	423	3	.....	.....
leaf length decreases	270	1	493	1	1034	1
leaf width decreases	268	0	509	3	1002	1
no. leaves decreases	284	1	525	1	1043	1
aggressiveness decreases	308	0	511	2	1039	1
size of culms decreases	259	3	465	1	939	1
no. culms decreases	265	2	540	1	1024	1
leaf earliness decreases	287	1	516	1	.....	.....
foliage color becomes blue-green	185	0	372	0	927	1
seed maturity becomes later	224	3	480	1	854	1
panicle type becomes closed	257	3	472	1	.....	.....
As late vigor decreases:						
early habit becomes tussock	319	1	541	3	995	1
late habit becomes tussock	219	1	417	3	.....	.....
leaf length decreases	246	1	479	1	1002	1
leaf width decreases	277	0	522	0	993	2
no. leaves decreases	263	1	513	1	1034	2
aggressiveness decreases	303	1	506	1	996	1
size of culms increases	229	0	459	3	1008	0
no. culms decreases	252	1	506	1	983	1
leaf earliness decreases	311	2	534	1	.....	.....
foliage color becomes blue-green	225	0	498	0	1004	1
seed maturity becomes later	223	3	467	3	998	1
panicle type becomes closed	242	3	496	3	.....	.....
As early habit becomes tussock:						
late habit becomes tussock	187	2	388	3	.....	.....
leaf length increases	215	0	444	0	1011	1
leaf width decreases	215	1	470	3	1021	1
no. leaves decreases	222	0	463	0	1011	1
aggressiveness decreases	235	1	448	1	999	1
size of culms decreases	198	2	422	3	1015	1
no. culms decreases	211	3	454	3	1003	1
leaf earliness becomes later	261	1	468	3	.....	.....
foliage color becomes blue-green	180	3	452	3	1009	1
seed maturity becomes earlier	193	3	440	0	1015	1
panicle type becomes closed	221	3	454	3	.....	.....

* *P* designations: *1*, indicated relationship highly significant (*P* exceeds .01); *2*, indicated relationship significant (*P* exceeds .05); *3*, tendency for indicated relationship (not significant); *0*, no association suggested.

TABLE 1—*Continued*

Character relations	Group 1				Group 2	
	a (normal)		*b* (aberrant)			
	No. plants	*P**	No. plants	*P**	No. plants	*P**
As late habit becomes tussock:						
leaf length decreases	132	3	286	1	.....	.....
leaf width decreases	142	1	355	3	.....	.....
no. leaves decreases	130	3	302	3	.....	.....
aggressiveness decreases	159	0	327	0	.....	.....
size of culms increases	106	1	254	1	.....	.....
no. culms decreases	126	1	312	2	.....	.....
leaf earliness becomes later	173	0	348	0	.....	.....
foliage color becomes blue-green	116	3	317	0	.....	.....
seed maturity becomes later	104	1	267	3	.....	.....
panicle type becomes closed	117	0	298	3	.....	.....
As leaf length decreases:						
leaf width decreases	172	3	409	2	1016	0
no. leaves decreases	151	1	335	1	1021	1
aggressiveness decreases	189	0	374	2	1018	2
size of culms increases	142	3	321	3	1024	1
no. culms decreases	160	3	356	2	1026	0
leaf earliness becomes later	197	3	358	3	.....	.....
foliage color becomes blue-green	141	3	366	0	1031	1
seed maturity becomes later	131	3	313	3	1038	2
panicle type becomes closed	148	2	329	3	.....	.....
As leaf width decreases:						
no. leaves decreases	177	0	421	0	897	1
aggressiveness decreases	206	3	409	1	1076	1
size of culms decreases	150	3	381	3	1016	1
no. culms decreases	174	2	438	3	1015	1
leaf earliness becomes later	220	3	440	3	.....	.....
foliage color becomes light green	155	3	411	3	989	1
seed maturity becomes earlier	148	0	388	3	1033	1
panicle type becomes closed	170	0	410	0	.....	.....
As no. leaves decreases:						
aggressiveness decreases	194	0	373	0	1038	0
size of culms decreases	141	3	334	3	1043	2
no. culms decreases	162	0	367	0	1028	1
leaf earliness becomes later	205	3	402	3	.....	.....
foliage color becomes blue-green	143	0	372	0	968	1
seed maturity becomes later	133	0	325	0	1038	1
panicle type becomes closed	156	3	346	3	.....	.....
As aggressiveness decreases:						
size of culms increases	170	0	344	0	1090	1
no. culms decreases	191	0	391	0	1008	1
leaf earliness becomes later	239	0	431	0	.....	.....
foliage color becomes blue-green	166	0	388	0	1009	1
seed maturity becomes later	170	3	358	3	1042	1
panicle type becomes closed	185	3	381	3	.....	.....
As size of culms increases:						
no. of culms decreases	136	1	332	3	916	1
leaf earliness becomes later	172	0	371	3	.....	.....
foliage color becomes blue-green	121	0	348	0	1011	1
seed maturity becomes later	106	3	294	1	1012	1
panicle type becomes closed	128	2	312	3	.....	.....
As no. culms decreases:						
leaf earliness becomes later	193	2	412	1	.....	.....
foliage color becomes blue-green	140	3	400	3	987	1
seed maturity becomes later	125	1	332	1	951	1
panicle type becomes closed	155	2	509	3	.....	.....

TABLE 1—*Continued*

Character relations	Group 1				Group 2	
	a (normal)		*b* (aberrant)		No. plants	*P**
	No. plants	*P**	No. plants	*P**		
As leaf earliness becomes later:						
foliage color becomes blue-green	166	3	398	3	.....	.....
seed maturity becomes later	182	2	387	0	.....	.....
panicle type becomes closed	190	0	408	0	.....	.....
As foliage color becomes blue-green:						
seed maturity becomes later	121	0	353	3	979	1
panicle type becomes closed	135	0	379	0	.....	.....
As seed maturity becomes later:						
panicle type becomes closed	117	3	307	3	.....	.....

strains are indicated. Primary characters desired in a forage strain of Kentucky bluegrass might include aggressiveness, leafiness, and abundant fruiting culms. The latter is related to seed production and subsequent propagation. Other highly desirable characters include the ability to develop vigorously early in the growing season and retain such activity over a long period.

The data are reviewed approximately in the order in which they are summarized in the table. One character is eliminated successively under each of the sections discussed in the tabular sequence. Statements made consist of a general interpretation of relations of the characters studied.

Plant height may be considered generally as an index of vigor during the vernal phase of plant development. Taller plants were lower in fall vigor, however, plants of intermediate height tending to be most vigorous. Likewise, the taller forms tended to be of tussock growth habit and to be aggressive. Strict tussock types would be less aggressive. Taller forms tended to develop more rapidly during the earlier portion of the growing season and to be more leafy and less stemmy in appearance. The texture, leaf width, and color of the leaves borne were quite variable, with taller plants tending to be more narrow leaved and light green in color. Tall types of plants also tended to have longer leaves, though short-leaved, tall types occurred. Negative relations existed between plant height and culm size, whereas that with the number of culms was positive. Late seed maturity was also characteristic of the taller types.

Early and late vigor will be considered together, since they were directly associated with each other, and their relations to other characters were essentially similar. Less vigorous, nonaggressive types were usually of tussock growth habit, their fewer leaves being shorter and frequently finer. Their culms were fine and may be few. Likewise, they were generally of late seed maturity.

With respect to early habit of growth the tussock type appeared to be associated with reduced aggressiveness and less markedly so with other characters including reduced leaf width, later leaf de-

velopment, and blue-green color. Size of culms appeared to decrease with the tussock growth habit. Number of leaves was relatively low for this type. Seed maturity appeared to be slightly earlier for tussock types. The relationship between classifications for early habit and late habit of growth is surprisingly low, though significant.

As late habit became more tussock in type, leaf width and aggressiveness were reduced. Size of culms tended to increase. Number and length of leaves, and lateness of seed maturity did not appear to be closely associated with late habit.

Data studied for the relation of leaf length to leaf width indicate that these characters were related though not closely. This is in general agreement with the relation observed by BÄR (1). There was a highly significant association between leaf length and leafiness, the longer-leaved types also appearing to have the most leaves. Likewise, as leaf length decreased, aggressiveness also decreased. Plants with long leaves tended to have fine culms. Essentially similar associations occurred between leaf length compared to leaf earliness, seed maturity, color, and panicle type.

No consistent association was found to occur between leaf width and number of leaves. A highly significant relation was determined for plants studied for these characters in group 2, indicating that as leaves became narrower they also became fewer in number. Narrow-leaved types were generally nonaggressive, but culm characters may be variable, both in size and in their number. In group 2, however, highly significant positive associations occurred. Narrow leaves were slightly associated with leaf lateness and light-green foliage color. Relations between leaf width and seed maturity were variable in the three plant groups studied. BROWN (2) found a positive correlation between leaf width and the number of florets and between leaf width and the number of rhizomes. Both sets of results appear to substantiate the general observation that aggressiveness is closely related to the number of rhizomes. The number of culms does not appear to be directly associated with the number of florets per spikelet, though this was not studied.

Leafy plants may be either aggressive or nonaggressive, no association occurring between these characters. Plants of this type that develop early in the spring later produce coarse culms that possess the closed type of panicle. No association was found in group 1 between leafiness and the number of culms, foliage color, or seed maturity. The data for group 2 indicate, however, that the number of leaves carried by a plant is positively related to numerous fruiting culms, light-green color of the foliage, and early seed maturity.

No association was found in group 1 between aggressiveness and culm size, number, or foliage color. The determinations made in group 2 are at variance with the group 1 data. The latter years' determination shows that nonaggressive types may have coarse and numerous culms and dark-blue-green foliage. Aggressiveness was not related to early development of leaves. Seed maturity was somewhat earlier and panicle type more closed for nonaggressive plants.

Coarseness may be accompanied by less numerous culms. In group 1*a*, however, a converse relationship is indicated. Such plants tend to develop late and may have a blue-green color, though no relationship is indicated in two of three comparisons. Coarse-textured plants give the

impression in the field of being of late seed maturity, and this relation is generally correct, though plants with coarse culms occasionally may be early in seed maturity. Field observations made in a cursory manner may also lead to incorrect interpretation of the latter character in plants of this type.

Frequently one may conceive of Kentucky bluegrass as possessing relatively numerous culms. This is often at error, plants with but few culms being relatively common among space-planted progenies of the species. In these studies plants late in the development of leaves usually tended to be blue-green rather than green in color. Their seed maturity was late, and plants had more closed panicles.

Plants that produced foliage late in the spring were not necessarily coarse and of dark-green color. Such an impression was derived earlier before detailed study. Seed maturity of such types was commonly late. Panicles of this type of plant were highly variable in appearance. As seed maturity became later, earliness of leaf growth decreased. Types maturing seed late often had panicles that were closed, but this association was not strong.

From these data it is evident that *P. pratensis* is, as delimited at present, a highly variable species, differing morphological types being found, owing to relatively free interrelationships of numerous characters. Although much variation occurs, with the exclusion of occasionally anomalous types the biotypes analyzed tend to fall into several rather broad, natural groups. These are essentially similar in their primary characteristics to those previously used to define the major subdivisions in several systematic treatments, but the writers will not discuss such groups at present. One large section includes plants with narrow leaves, small florets, and green foliage. Another complex has large florets and blue-green color. Other diagnostic characters were not considered in these studies.

Summary and conclusions

1. Approximately five thousand plants selected as generally representative of *Poa pratensis* were studied for fifteen different characters. Their paired associations with one another were determined.

2. Early vigor and late vigor were directly associated, and both were related to leaf earliness. Their incorporation in a strain might thus be readily accomplished. Likewise, they are positively associated with aggressiveness and leafiness. These early, vigorous types are generally aggressive and leafy. There were also positive relationships between leaf length and leaf width, plant vigor, and leafiness. Early types were generally not long leaved. The data indicate that leaf length is not closely associated with leaf width. However, in observing many plants in the field one obtains the impression that width and length of leaves are negatively associated. Usually, however, long leaves tend to be narrow, and wide leaves tend to be short. This tendency, which is not strong, is in general agreement with the findings of BÄR (**1**). There was no association between aggressiveness and leafiness. Early, vigorous types usually had numerous culms, whereas short, numerous leaves were negatively associated with culm number. A positive relationship was found between the number of culms and aggressiveness, in one of three groups studied. BROWN (**2**) found a positive correlation between leaf width and number of florets and between leaf width and the number of rhizomes. Present studies indicate a negative relation

between leaf width and the number of culms and a positive association of leaf width with aggressiveness.

3. Other associations may be determined from the data presented. Average relationships obtained suggest that certain features may be difficult to combine in a given strain. For example, fine-textured culms and leaves may be associated with the tussock growth habit and less leafiness. Likewise, tussock types appear somewhat later in leaf earliness. Although these associations exist, occasional plants may possess character combinations in direct exception to the general relations indicated.

4. The lack of agreement among groups with respect to many of the relationships studied is of interest. This would appear to suggest that such relationships were not close and that the occasional significant F values obtained may be a result of errors of random sampling and possible bias which the type and extent of populations studied and methods used have not overcome.

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HYBRID VIGOR IN A TOMATO CROSS

W. GORDON WHALEY

Introduction

Interest in hybrid vigor stems partly from the agricultural potentialities of outstanding plant and animal hybrids and partly from the fact that such hybrids presumably represent good experimental material for the study of certain gene actions. Although one of the first steps in a search for the biological basis of such a phenomenon as hybrid vigor should be a clear definition of its character, there have been few attempts to describe hybrid vigor in any but the most general terms. Most investigators have merely pointed out that the hybrids under consideration were larger or more fruitful than the types crossed to produce them.

Obviously, the attainment of larger size or the production of more fruit need involve only time differences in reaching maturity or full fruitfulness. Studies in our laboratory have indicated that some hybrids considered to be particularly vigorous differ from one or both parental types mainly in developing somewhat more rapidly and producing the bulk of their fruit earlier. If such cases are eliminated and if we consider only those plants which ultimately outgrow or outproduce their parents as showing hybrid vigor, there are still a number of other considerations involved.

A hybrid may gain size advantage by having a higher postgermination growth rate, by germinating relatively more rapidly and then growing at essentially the same rate as one or both of its parents for approximately the same length of time, or by having, as ASHBY (1) put it, greater "initial capital."

Reference to the possibilities for the production of hybrid vigor suggests that it is unlikely that all instances can be explained in accordance with a single pattern. It does seem clear, however, that the investigation of certain cases of hybrid vigor might be expected to add considerably to our knowledge of the character of genetic differences and the manner of gene actions.

This paper deals with the results of an attempt to establish the nature of the hybrid advantage in one tomato cross. Weight advantage was taken to indicate heterosis. The pattern of weight increase was followed, as were certain general morphological features. Total phosphorus and nitrogen content, percentage of carbohydrate, total proteolytic activity, and catalase activity of the shoot apices were determined for plants of successive ages.

Material and methods

The tomatoes used were University of Texas lines 103 and 154 and their hybrid, 103 × 154. Line 103 is a selection from *Lycopersicon esculentum* Mill. variety Yellow Plum. Line 154 is a selection from *L. pimpinellifolium* Mill., the Red Currant tomato. Seeds of both these lines were originally obtained from commercial sources, but the lines have been inbred for several years. This particular cross does well under the field conditions of central Texas, and it represents a combination of two relatively diverse types.

The plants for the main part of the experiment were grown in a screened lathhouse at Austin, Texas, beginning early in May. Seeds were planted in lots of twelve in 2-gallon crocks containing a mixture of washed quartz sand and vermiculite (expanded mica). Nutrients were supplied in a solution prepared according to Formula IV of ELLIS and SWANEY (**3**). Growth of the plants was good and apparently normal in all respects, although they were a slightly darker green color, and the stems were slightly more elongated, than those of field-grown plants of the same lines. These differences resulted from the partial shading by lath and screens or from nutritional differences between the plants in the house and those in the field.

Samplings were made at weekly intervals. At each early sampling period twenty apparently typical plants of each line were selected for measurement and analysis. At these times any atypical plants appearing in the cultures were eliminated, and an effort was made through the whole course of the experiment to keep, by selective elimination, uniformity among the plants. At later sampling periods, when the plants had become too large for handling twenty at a time, the number in a sample was reduced, first to ten, then to six, and finally to two. The plants selected were removed from the crocks with as much of the root systems as could be obtained without taking special care to preserve the attachment of the smallest roots. Half the plants in a sample were used for morphological and growth analyses, the other half for chemical analyses.

All samples were taken just after sundown to insure, in so far as possible, uniformity with respect to moisture content and synthesis-translocation factors. Measurements and fresh-weight determinations were made immediately. For dry-weight determinations the plant parts were dried to constant weight at 100° C.

For phosphorus and nitrogen determinations the leaves, stems, and roots were dried separately in a forced-air drying oven at 85°–90° C. and then weighed. Either the whole sample or, in the case of large samples, a portion of the sample which had been ground in a Wiley mill to pass through a 60-mesh sieve was taken for analysis.

For phosphorus determinations samples were wet-ashed on a hot plate with concentrated sulfuric acid and 30% hydrogen peroxide, following WOLF's (**15**) procedure. The solution was then made up to 50 ml. with MORGAN's universal extracting solution (**8**). Appropriate aliquots (5–8 ml.) of the plant extract were taken for total phosphorus determination by the FISKE-SUBBAROW method (**4**), using a Cenco-Sheard photelometer and a blue filter. Photometric readings were converted into micrograms of phosphorus with the aid of a standard curve prepared with known amounts of phosphorus.

For Kjeldahl nitrogen determinations a direct Nesslerization procedure according to KOCH and MCMEEKIN (**5**) followed by photocolorimetric estimation was employed. Four-milligram samples were weighed into large Pyrex test tubes calibrated to 10 ml. of 1:1 sulfuric acid. A few drops of 30% hydrogen peroxide were added when the contents of the tubes were almost clear. After digestion was complete, distilled water was added up to the 10-ml. mark. Ten milliliters of the KOCH-modified Nessler-Folin solution were then added to develop color. Readings were taken through a green filter 10 minutes after addition of the reagent. The standard curve was prepared by using a series of dilutions of the

standard solution of ammonium chloride.

For carbohydrate analyses the samples were weighed, dried to constant weight in a drying oven, and reweighed. Hot distilled alcohol containing sufficient precipitated calcium carbonate to neutralize the acidity was then added to each weighed sample in an amount sufficient to bring the final concentration of alcohol to an estimated 80%. The alcohol mixture was heated on a water bath for 30 minutes, during which it was stirred frequently. Samples were cooled and stored until analysis. Sugar analyses were made by the MUNSON and WALKER method for reducing sugars (**10**), and starch analyses by the acid hydrolysis method (**6**).

Determinations of catalase activity were made in the following manner. The apical 5-mm. portions of the shoot tips (including the shoot apical meristem and associated young leaf primordia) were cut from the main shoot and the side branches of the sample plants. These segments were cut into small pieces with a sharp scalpel, and the pieces were thoroughly mixed. After the mixing, duplicate samples of 9.5 gm. of the chopped tissue were weighed out and ground with a small amount of water and an excess of calcium carbonate until reduced to a fine pulp. This pulp was transferred to graduated tubes, and each sample was made up to the 10-ml. mark. Duplicate aliquots of 2–5 ml. were taken for each determination. Catalase activity was then determined by the release of oxygen from hydrogen peroxide (**6**).

Total proteolytic activity was determined by the method of MOUNFIELD (**9**), modified in that cysteine was used as activator, thymol sulfonthalein as indicator, and the initial gelatin concentration was 1% instead of 2%. Activity is reported as increase in ml. $N/20$ sodium hydroxide per gm. fresh weight per 5-ml. aliquot of digestion mixture after a 24-hour digestion at 40° C.

Results and discussion

The average air-dry weights of 200 seeds in milligrams were: line 103, 2.2 ± 0.04 (standard error); line 154, 0.9 ± 0.02; hybrid 103×154, 2.0 ± 0.03. The hybrid did not have a seed-weight advantage. No attempt was made to compare the embryos as to either weight or size. Fresh and dry weights of both inbreds and the hybrid at the various sampling periods are shown in table 1. It is clear from these data that the hybrid had an advantage in both fresh weight and dry weight through the entire period of measurement. Line 154 was the smaller of the inbreds in terms of either fresh or dry weight. Line 103 occupied a position intermediate between the smaller inbred and the hybrid. Growth curves (not given) based upon the data in table 1 indicate that during the period studied the hybrid growth rate did not significantly exceed that of the heavier inbred, line 103. However, since the hybrid grew as rapidly as line 103, the advantage established during early seedling growth was maintained. This observa-

TABLE 1

WEIGHT OF PLANTS OF SUCCESSIVE AGES (GM.)

AGE IN DAYS	LINE 103		LINE 154		HYBRID 103×154	
	Fresh	Dry	Fresh	Dry	Fresh	Dry
11	0.2	0.02	0.08	0.01	0.3	0.02
18	1.3	0.08	0.53	0.05	1.9	0.12
25	4.4	0.27	1.02	0.07	6.5	0.43
31	8.8	0.57	3.92	0.27	15.8	1.12
45	22.4	1.50	11.23	0.92	39.5	3.42
52	46.3	3.98	11.70	0.90	59.2	5.57
59	95.8	10.49	48.91	4.03	115.1	12.29
66	107.5	10.41	96.20	10.19	116.3	13.38
73	108.7	15.90	.....	.....	187.7	24.86

tion is in agreement with the earlier findings of ASHBY (1) and WHALEY (12).

ASHBY (1) reported the hybrid's advantage in a tomato cross to include height of the plant. He noted that height is an unsatisfactory characteristic for evaluation of hybrid vigor but included the measurement in his experiment because of the frequency with which a height advantage has been accepted as demonstrating the presence of heterosis. In our experiment total length of the stems and branches was measured at each of the sampling periods, partly to check upon presence or absence of height heterosis and partly as a means of recording growth-pattern differences of the plants.

Stem lengths, number of branches, branch lengths, leaf numbers, and the branch position of hypothetical average plants are shown in figure 1. The dia-

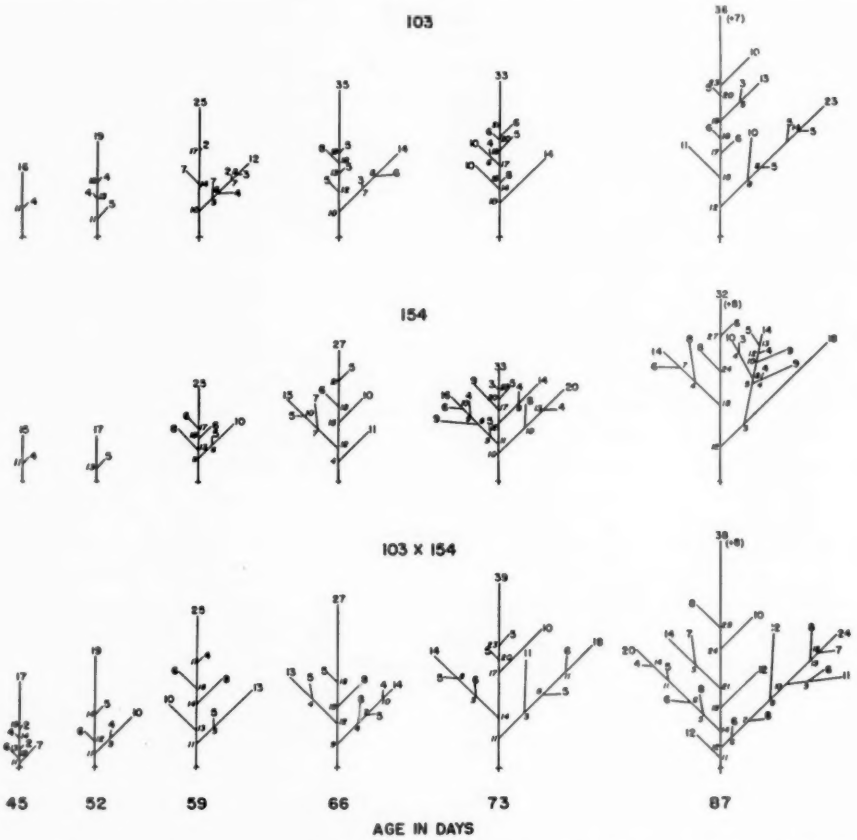

FIG. 1.—Diagrams showing number and position of branches and number of leaves of tomato plants of both inbreds and hybrid at successive sampling periods. Figures at end of each line represent number of leaves borne on that particular branch. Figures at bases of lines represent number of leaf at which the branch occurred most frequently in that particular sample. 1 mm. of line = 2.4 cm. of stem. (For further explanation see text.)

grams of the figure were constructed on the basis of the average of the plants in each sample. No attempt was made, in constructing the diagram, to depict the fact that according to most observers the main axis of a tomato plant is constituted of a series of successive principal axes developed from axillary buds. The same pattern holds for the lateral axes. Where the point of origin of a branch differed among the plants of a sample, the diagrams were drawn to show the branch where it was most frequently found in that particular sample set of plants. There clearly was no rigidly fixed pattern of branch origin, a fact already noted by WENT (11) and others. Despite the variation within lines, there were, however, definite differences among the lines, already apparent in the 45-day-old plants.

It is clear from the diagrams that the hybrid advantage in this particular cross can be interpreted in terms of the development of a greater mass of stem tissue supporting more leaves and distributed over a significantly larger number of branches.

Eleven-day-old plants had average leaf numbers as follows: line 103, 3.7; line 154, 3.8; and the hybrid, 4.4. Average leaf numbers for 25-day-old plants were 9.4 for line 103, 8.1 for line 154, and 10.8 for 103 × 154. The leaf-number advantage of the hybrid was thus established early in development. This could result only from greater activity of the shoot apical meristems of the hybrid in the formation of leaf primordia. This advantage was maintained to maturity. Within this pattern the apical meristem of the main shoot produced more leaves in the hybrid than in either of the inbred lines.

With relation to total photosynthetic efficiency, consideration has to be given not to leaf number but to total area of leaf surface. With this in mind, at each sampling period an apparently typical plant was selected from each group, harvested, and used for leaf-area determinations. The leaves were removed separately, the outline of each was traced on paper, and the area within the individual tracings determined with a planimeter. Total leaf area was thus determined for representative plants. The hybrid showed a consistent advantage in total leaf area, and as the plants became older, this advantage tended to increase. For example, at 45 days the leaf areas in square centimeters were: line 103—348, line 154—273, hybrid 103 × 154—625; at 87 days, 103—4,140, 154—2,980, 103 × 154—5,110. Of the inbreds, line 103 was closest to the hybrid. The advantage of the hybrid derived from a combination of leaf number and area per mature leaf.

No attempt was made to follow the development of flowers and fruits, but the first visibly apparent inflorescences in line 103 were found in 38-day-old plants, in line 154 at 45 days, and in the hybrid plants at 31 days. The flower buds of the hybrid thus reached a visible stage some 7 days earlier than did those of the earlier flowering inbred parent. Data on fruit size and number were not recorded in this experiment. From separate observations on this same cross it may be noted that the hybrid fruits are intermediate between those of the two inbred lines as to volume and weight and that the considerably heavier fruit production of the hybrids is a result of formation and development of a larger number of fruits rather than an advantage in individual fruit size.

The comparison of the morphological patterns of the inbreds and the heterotic hybrid suggests greater meristematic activity as the immediate source of the hybrid advantage. During the early post-

embryo growth period the hybrid definitely formed tissues and structures at a more rapid pace, and, as the plants developed, the formation of more meristematic areas in the hybrid gave it a further advantage in the laying-down of tissues and new structures. The end differences were size differences, but it seems best to interpret them as the results of differences in total meristematic activity. The establishment of such differences between the inbreds and the hybrid raises the question of the nature of the physiological differences of which these morphological and size differences are manifestations.

A generally higher rate of physiological activity in the hybrid might be reflected in such basic considerations as total nitrogen content or total phosphorus content or total starch formed per unit of tissue.

Total phosphorus content, on a μgm/mg dry-weight basis, of both inbreds and the hybrids at four of the samplings is shown in table 2. The figures do not indicate any significant differences between the inbreds and the hybrid as to total phosphorus content of the leaves, the stems, or the roots. In the full data there are indications of some changes in phosphorus concentration as the plants matured. The older plants had relatively less phosphorus in the leaves, somewhat more in the stems and roots.

Although there was somewhat more variation in nitrogen content than in phosphorus content, the data (table 2) show that the total nitrogen content of the leaves increased somewhat as the plants matured. Stem nitrogen showed a more consistent pattern, with a drop from the initial level to a minimum at about the time inflorescence development became strong and the first fruit-set was beginning. This minimum level was succeeded by an increase. In the 87-day-old plants the nitrogen content of the leaves and the roots was of approximately the same order of magnitude, the

TABLE 2

TOTAL PHOSPHORUS AND TOTAL NITROGEN CONTENTS IN INBRED AND HYBRID TOMATO PLANTS AT DIFFERENT AGES

Line	24 days				45 days			59 days			87 days			
	Leaves	Stems	Roots	Cotyledons	Leaves	Stems	Roots	Leaves	Stems	Roots	Leaves	Stems	Roots	Flowers
	Total phosphorus (μgm/mg dry weight)													
103	5.1	2.9	3.0	.....	5.3	2.5	3.5	3.8	3.1	3.6	3.6	2.8	3.9	.....
154	5.0	2.8	3.1	.....	5.8	3.1	2.7	3.1	2.8	3.2	3.5	4.1	4.1	.....
103×154	4.8	2.6	2.5	.....	4.7	4.2	1.1	3.5	2.7	3.2	3.7	3.4	3.7	.....
	Total nitrogen (% of dry weight)													
103	4.0	1.5	.....	3.3	2.3	0.8	1.8	3.5	0.9	2.0	4.6	2.0	4.4	5.6
154	2.6	2.0	.....	3.4	3.3	0.8	1.7	3.4	0.9	1.8	4.5	2.3	4.2	6.3
103×154	3.3	2.0	.....	8.5	2.3	1.0	1.3	2.8	1.0	2.5	4.5	2.0	4.8	5.8

stems had about half this amount of nitrogen per unit of dry weight, and the flowers had considerably more than the leaves or the roots had.

The data on starch content (table 3) suggest that the hybrids may have had a slight advantage in starch content of the leaf during the early stages of development, although over the whole growth period they had no advantage. In general, the stems had approximately twice as much starch as the leaves.

TABLE 3

STARCH (% OF DRY WEIGHT) IN INBRED AND HYBRID TOMATO PLANTS AT DIFFERENT AGES

Line	24 days		45 days		59 days		87 days	
	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems
103	2.3	4.8	2.0	8.9	2.7	6.1	3.3	5.8
154	2.4	5.2	2.1	5.3	3.1	5.0	3.9	8.1
103×154	2.5	7.1	2.6	4.8	2.5	4.7	3.8	7.4

Catalase-activity determinations were made on stem-tip samples of 66-, 73-, and 87-day-old plants (table 4). In the case of the 66-day-old plants, however, only the later-formed branch apices were analyzed. The catalase-activity figures indicate that in this characteristic, at least, the hybrid plants did have a clear-cut advantage. These apparent differences in catalase activity should be examined, though, with the growth-pattern differences in mind. The 73- and 87-day determinations were made on sample material which was a composite of all the shoot meristems of the sampled plants. Reference to the branching-pattern diagrams will indicate that there were marked differences in the number of stem meristems on the different plants at 73 and 87 days. These differences in numbers would not necessarily be significant, but the branching diagrams suggest that more of the stem meristems of the hybrids were in a highly active growth phase than was the case with either of the inbreds. If high catalase activity parallels rapid growth and development of the stem apices, then a greater proportion of actively growing stem apices might be responsible for the apparent differences in catalase activity,

TABLE 4

CATALASE ACTIVITY OF STEM TIPS (ML O_2/MIN/GM FRESH WEIGHT)

Line	66 days*	73 days	87 days
103	72.2	53.7	50.0
154	33.4	25.8	18.0
103×154	97.8	63.8	78.3

* Young shoot apices only.

that is, the apparent differences could be nothing more than a reflection of differences in total meristematic activity at the time of sampling. That this is not the explanation for a major part of the differences is indicated by the figures for the 66-day sampling, which represent the catalase activity of the tips of only actively growing branches.

Determinations of total proteolytic activity (fig. 2) were made on germinating seeds and on young seedlings to determine whether activity levels of other enzyme systems might be involved in the early developed differences. Total proteolytic activity is, of course, a combina-

tion of a number of enzyme processes and, therefore, only a general expression of a group of metabolic activities. The data do not reveal the relationship of any single process or group of processes to the hybrid advantage, but they do give an indication of pattern of early development of enzyme activity, and they suggest slight differences between the heterotic hybrids and the inbreds. Interpretations of the apparent differences would have to be preceded by an adequate measurement of their significance and an investigation of the particular reactions involved.

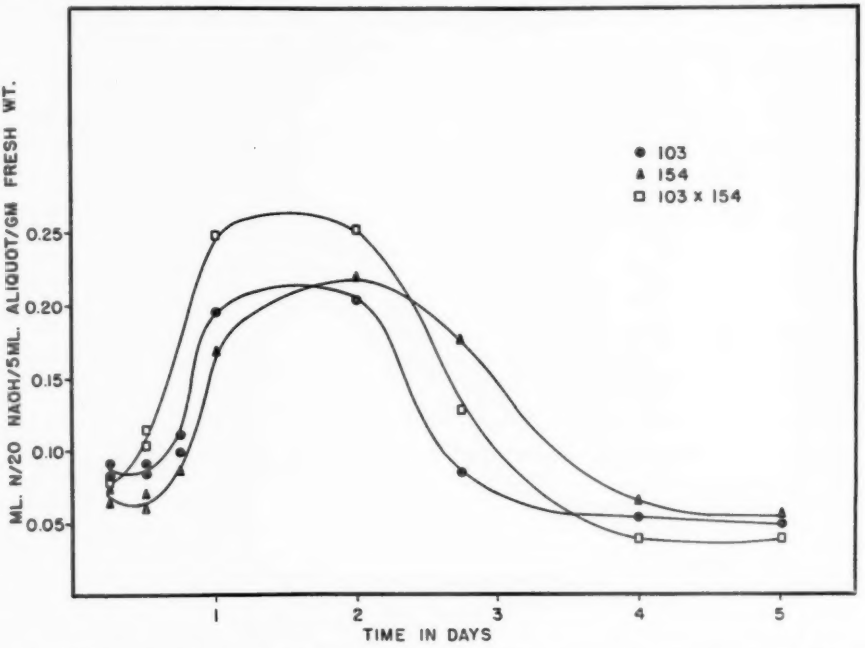

FIG. 2.—Total proteolytic activity of germinating seeds and young seedlings of tomato lines 103, 154, and hybrid 103 × 154. Values represent increase in ml. $N/20$ NaOH per gm. fresh weight per 5-ml. aliquot of digestion mixture after 24-hr. digestion at 40° C.

The results suggest that the expression of heterosis in this particular tomato cross follows the pattern described by ASHBY (1) and his co-workers (7) for other tomato crosses and by WHALEY for several corn crosses (14). The advantage of the hybrid is developed early and maintained consistently to maturity. There is no evidence of greater metabolic efficiency of the bulk of the hybrid tissues throughout the greater part of the growth cycle. Inasmuch as the hybrid does not have a seed-size advantage, we must look in the germination and early postgermination stages, and perhaps to the activities of the meristematic regions, for the differences responsible for the development of the hybrid vigor.

Differences which are of importance during the germination and early postgermination stages could hardly be related to factors of absorption and general translocation, although the movement of

food into developing embryos does appear to be of importance in some instances of hybrid vigor (**14**). The occurrence of differences in catalase activity and of differences in the pattern of protease activity in the developing seedlings suggests that the elaboration and subsequent activity of various enzyme systems may represent the phase of metabolism in which the differences responsible for heterosis should be sought.

Many of the so-called "biochemical mutations" reported in lower organisms (**2**) involve deficiencies in the production or utilization of substances whose activities relate to specific steps in enzyme synthesis or enzyme actions. If, as has been postulated (**13**), the occurrence of heterosis is a result of allelic combinations which have the effect of covering up accumulated deleterious recessive alleles, it would not seem unreasonable to assume that mutations of a similar character are concerned in the development of heterosis.

This postulate tends, at least, to define a time period in the development of the organism and, within that time period, a particular group of reactions and processes as subject matter for investigations aimed at elucidating the bases of hybrid vigor. If the experimental conditions can be sufficiently well controlled and the complications of photosynthesis and subsequent processes eliminated or circumvented, the investigation of whether differences in genetic constitution involve any of the already recorded types of biochemical mutations might well prove profitable.

Summary

1. Growth and development of two inbred lines of tomato and their hybrid were followed with respect to increase in weight, increase in height, differences of branching pattern, total phosphorus content, total nitrogen content, total starch content, catalase activity of the stem tips, and total proteolytic activity of germinating seeds and seedlings.

2. The morphological data indicate an early established and subsequently maintained size advantage for the hybrid.

3. No significant differences in total nitrogen, total phosphorus, or starch content were recorded.

4. The hybrids appeared to have higher catalase activity in stem tips, and there definitely were differences in the pattern of development of total proteolytic activity.

5. The results suggest that in certain instances the investigation of the bases of heterosis should be confined to the early growth stages and should deal with differences in the development of the enzyme systems and their activity.

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PLANT RESEARCH INSTITUTE
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AUSTIN, TEXAS
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CYTOCHEMICAL STUDIES ON PYCNOTIC ROOT-TIP CELLS

NEBAHAT YAKAR[1]

Introduction

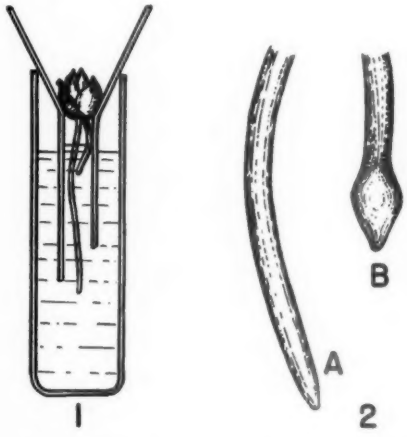
After it had been demonstrated that colchicine causes mitotic disturbances (**3**) and induces polyploidy (**1**), many other investigators reported mitotic disturbances and chromosomal aberrations in chemically treated cells. The study reported here was undertaken to determine cytochemically whether such aberrations involve detectable alterations in the constitution and patterns of association of nucleic acids and proteins. Root tips of lily (*Lilium tigrinum* L.) and onion (*Allium cepa* L.) were treated with various chemicals and then were fixed and sectioned for cytochemical analysis involving enzymatic hydrolysis under the conditions specified by KAUFMANN *et al.* (**5**).

[1] This study was completed while the author was working at the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, New York, as a Fellow of Istanbul University. Present address: Department of Genetics and Pharmacobotany, Istanbul University, Süleymaniye, Istanbul, Turkey.

Material and methods

Bulbils of lily were germinated on moist filter paper in Petri dishes at room temperature (*ca.* 22° C.). When the roots had grown to a length of about 2–3 cm., the bulbils were transferred to small funnels inserted in shell vials containing solutions of the chemicals to be tested (fig. 1). Bulbs of onion were grown over the mouths of small beakers filled with tap water, until the roots had attained a length of 1–2 cm., and were then transferred to similar vessels containing the solutions to be tested. The lily roots were treated for 24 hours, the onion roots for 24, 48, or 72 hours. After treatment, the roots were washed free of the chemical, and the tips were amputated, fixed in Carnoy's acetic acid–alcohol mixture (1:3), transferred to 70% ethyl alcohol, hardened in chloroform, dehydrated in graded alcohols, cleared in xylene, imbedded in paraffin in groups of two or three, and cut into 10 μ sections. Some

sections were stained with methyl green–pyronin for preliminary examination of morphological disturbances produced by the chemicals. Other sections from the same roots were digested, before being stained, in solutions of crystalline enzymes such as ribonuclease, desoxyribonuclease, and pepsin, in an effort to determine the nature of the cytochemical changes effected by the chemicals being tested. These digested tissues, and those of undigested controls, were stained in the following dyes: methyl green and pyronin alone or in combination, fast green, and the leucobasic fuchsin of the Feulgen reagent.



FIGS. 1, 2.—Fig. 1, method of treating roots of *L. tigrinum* with chemical solutions. Fig. 2, c-tumor formation in root of *L. tigrinum*, treated with 0.001 *M* colchicine for 24 hours. *A*, control; *B*, c-tumor formation. ×4.

The chemicals used in these experiments were colchicine, caffeine, hydroquinone, pyrogallol, pyrocatechol, and *p*-aminodimethylaniline monohydrochloride in 0.01 and 0.001 *M* concentrations; and 8-hydroxyquinoline in 0.005 and 0.001 *M* concentrations. These chemicals (with the exception of *p*-aminodimethylaniline monohydrochloride) had previously been shown to effect mitotic disturbances and chromosomal aberrations (**2, 7, 10, 11, 15**).

Observations

MORPHOLOGICAL CHANGES

The morphological changes induced in root tips by the chemical treatments may be grouped into six classes:

1. C-TUMOR FORMATION.—Root tumors produced by chemical treatment involve an increase in all dimensions of the meristematic cells. Only colchicine-treated roots showed c-tumors in these experiments (fig. 2).

2. C-MITOSES.—These are abnormal mitotic figures, in which the spindle is inactivated and the contracted chromosomes are scattered throughout the cytoplasm in the form of closely appressed pairs, known as "c-pairs" (fig. 3*C*). The mitotic tissues of root tips treated with 0.01 and 0.001 *M* colchicine, and 0.001 *M* hydroquinone, or pyrogallol, for 24 hours, showed c-mitoses.

3. CHROMOSOME FRAGMENTATION.—The action of the chemical leads to chromosome breakage. Fragments could be detected at anaphase and telophase, lying free in the equatorial region of the cell (fig. 3*D*, *E*); it was difficult to detect them during earlier phases. Chromosomal fragments were observed in the root-tip tissues treated with 0.001 *M* caffeine, hydroquinone, or pyrogallol for 24 hours.

4. FAILURE OF CELL-PLATE FORMATION.—Inhibition of cell-plate formation (although nuclear division occurs) results in binucleate cells (fig. 3*F*). Only root tips treated with 0.01 *M* and 0.001 *M* caffeine showed this phenomenon.

5. INCREASE IN SIZE OF NUCLEOLUS.—The nucleoli of cells of all chemically treated roots were significantly larger than those of untreated roots; the aver-

age diameter of the nucleoli in root tips of *L. tigrinum* treated with 0.001 *M* colchicine was 3.38 ± 0.07 μ, whereas in controls it was 2.23 ± 0.05 μ.

6. AGGLUTINATION OF CHROMATIN.—Some cells were characterized cytologically by agglutination of chromatin, and just named for 48 or 72 hours. Agglutination of chromatin may be regarded as one aspect of the phenomenon of pycnosis. In pycnotic cells the nuclei were significantly smaller than those of nonpycnotic cells; the average diameter of the nuclei in root tips of *L. tigrinum* treated with

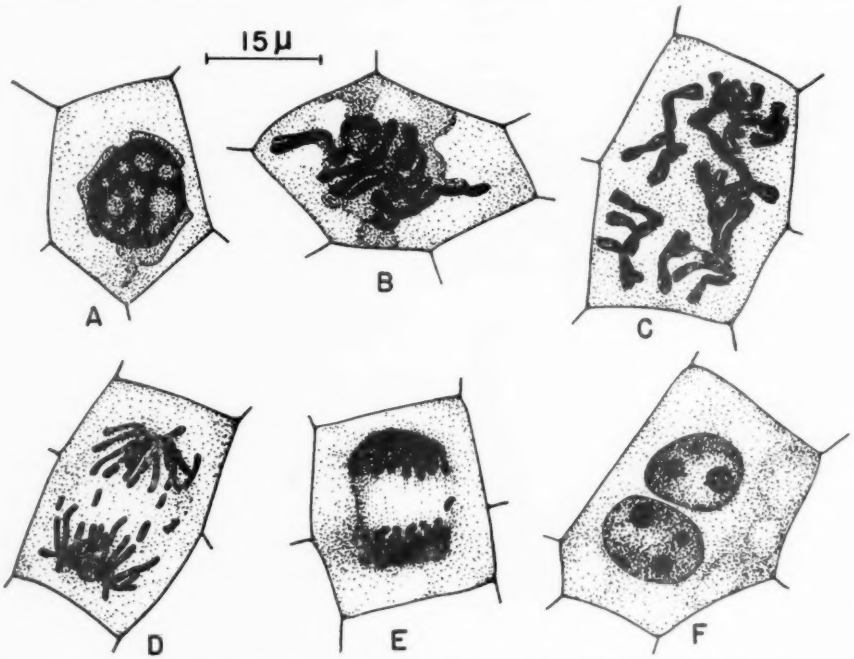


FIG. 3.—Cytological changes induced in root tips of *L. tigrinum* by treatment with 0.01 *M* hydroquinone (*A*, *B*), 0.001 *M* colchicine (*C*), 0.001 *M* pyrogallol (*D*), and 0.001 *M* caffeine (*E*, *F*) for 24 hours. *A*, *B*, cells showing agglutinated chromatin and accumulations of extracted materials around nucleus (*A*), or around dividing chromosomes (*B*); *C*, c-mitosis; *D*, *E*, chromosome fragments on spindle; *F*, binucleate cell formed as consequence of failure of cell-plate formation.

this change was reflected macroscopically in softening of the tissues of the roots. Agglutination was observed in cells of roots treated for 24 hours with 0.01 *M* hydroquinone, pyrogallol, or *p*-aminodimethylaniline monohydrochloride; with 0.01 or 0.001 *M* pyrocatechol; or with 0.005 or 0.001 *M* 8-hydroxyquinoline; and in all roots (except those treated with caffeine) exposed to the chemicals 0.01 *M* pyrocatechol was 10.23 ± 0.19 μ, whereas in controls it was 13.28 ± 0.24 μ.

CYTOCHEMICAL CHANGES

No differences were found between cells of chemically treated roots and those of untreated roots with respect to stainability or digestibility of nucleic acids and proteins, unless the chemicals had caused agglutination of chromo-

somal materials. For this reason, description of the cytochemical findings will be restricted to the pycnotic cells (excluding those produced by treatment with 0.01 *M* pyrocatechol, which were highly resistant to enzymatic hydrolysis).

When pycnotic cells were stained with methyl green–pyronin, cytoplasm and nucleoli colored a bright red, somewhat more intense than in nonpycnotic cells, and nuclei stained a more or less uniform dark violet. The red color was due to stainability with pyronin, the violet presumably to the presence of materials stainable with both pyronin and methyl green. In some cells a deep-red-staining substance had accumulated in the nucleoli, which were larger and more numerous than in nonpycnotic cells, or it surrounded the nuclei, from which it appeared to have escaped (fig. 3*A*). A comparable dislocation of materials apparently had occurred during division stages, as the condensed chromosomes in some cells were surrounded by pools of material that stained deep red. As a result the chromosomes lost their individuality and appeared as aggregates (fig. 3*B*). Nevertheless, they gave a positive Feulgen reaction.

These color patterns were not altered by digestion of the cells in ribonuclease (0.1–0.5 mg/ml) for 1 hour at 37° C. at pH 6.0, whereas the same treatment of cells that had not been chemically treated completely reduced the pyronin stainability of cytoplasm, nucleoli, and chromatin. Digestion of pycnotic cells in ribonuclease at a concentration of 1 mg/ml for 8–24 hours reduced or eliminated pyronin-stainability of cytoplasm. Stainability of nucleoli, chromatin, and perinuclear areas was in general decreased, although in some cells these structures retained their affinity for the dyes and showed violet-colored nuclei with dark-red-staining nucleoli and perinuclear areas.

Desoxyribonuclease in a concentration of 0.001 mg/ml in 0.003 *M* $MgSO_4$ at pH 6.0 for 2 hours at 37° C. did not reduce stainability of these pycnotic nuclei with methyl green (when stained in methyl green–pyronin), although methyl-green stainability was eliminated by this enzyme when used under the same conditions on nonpycnotic cells. Desoxyribonuclease in this concentration also was ineffective in reducing pyronin stainability of the pycnotic cells. A higher concentration of the enzyme (0.01 mg/ml) eliminated practically all methyl-green (and Feulgen) stainability from agglutinated cells. This same concentration also markedly reduced pyronin stainability of the chromatin but did not appreciably alter the stainability of cytoplasm, nucleoli, and perinuclear spots. Only slight reduction of pyronin stainability of chromatin by desoxyribonuclease was observed in nonpycnotic nuclei.

On the other hand, pepsin (1 mg/ml in 0.02 *N* HCl for 2 hours at 37° C.) greatly reduced pyronin stainability of both nucleoli and perinuclear spots in the pycnotic cells, whereas their stainability was not reduced by 0.02 *N* HCl when used under the same experimental conditions. Pyronin stainability of cytoplasm and chromatin was not impaired by digestion of the cells in pepsin. In contrast with these results, treatment of nonpycnotic cells with pepsin increased stainability of cytoplasm and chromatin with pyronin.

When pepsin (1 mg/ml) and ribonuclease (1 mg/ml) were used in either of the two possible sequences (each for 2 hours at 37° C.), cytoplasm, nucleoli, and perinuclear spots did not stain with pyronin, but the stainability of chromatin was not impaired.

Prolonged treatment (for example, 48–72 hours) with 0.01 *M* hydroquinone and 0.005 *M* 8-hydroxyquinoline apparently caused further dislocation of cellular materials. When stained with methyl green–pyronin, cytoplasm and nucleoli showed a faint pink color, the chromatin either faint pink or pale violet. The Feulgen reaction of these nuclei was also weak or negative. Cells near the periphery of the root (especially after treatment for 72 hours with 8-hydroxyquinoline), whose nuclei stained feebly or not at all with methyl green, sometimes showed blue chromonema-like threads in the cytosome. These threads were also Feulgen-positive. Treatment with ribonuclease altered pyronin stainability of the threads (when stained with methyl green–pyronin) so that they stained green; and treatment with desoxyribonuclease markedly reduced or eliminated stainability with methyl green and the Feulgen reagent. After such elimination of methyl-green stainability the threads were not clearly differentiated from the surrounding cytoplasm when stained in methyl green–pyronin, but they stained with pyronin when this dye was used alone on cells digested in desoxyribonuclease.

Discussion

When the chemicals tested were used for short periods of time in low concentrations, they produced chromosomal aberrations and mitotic disturbances. When used in higher concentrations or for longer periods of time, they caused pycnosis with characteristic agglutination of chromatin. The concentration and length of exposure required to produce such toxic effects were found to depend on the chemical used.

The types of pycnotic degeneration observed resemble in many respects those observed by other workers in malignant tissues and in cells treated with radiations and chemicals (review of literature in **9**). Pycnosis is characterized by shrinkage and intense, uniform staining of the nucleus, loss of individuality of chromosomes, and the eventual dislocation of these materials. The present study has attempted to determine cytochemically the nature of the changes involved.

Analysis is complicated by the fact that after treatment of roots with some chemicals, very high concentrations of enzymes were required to effect degradation of cellular materials. An extreme case was afforded by exposure to 0.01 *M* pyrocatechol; even high concentrations of the enzyme then failed to alter stainability of cellular materials. Such results may have been due to the action of the chemicals in altering cellular substrates or in inhibiting enzymatic activity. It is well known that different kinds of fixatives affect the capacity of enzymes to digest cells (**4, 6, 12, 14**). The evidence presented here suggests that the action of enzymes on root-tip cells may have been limited by the chemicals used in treating the roots. In those preparations in which digestion was effected, however, it was possible to draw some conclusions about the nature of the cellular materials involved.

There seems little question that the chromonema-like threads found in the cytoplasm of some pycnotic cells contain polymerized desoxyribonucleic acid (DNA), since they stain with both methyl green and the leucobasic fuchsin of the Feulgen reagent before treatment with desoxyribonuclease but not after such treatment. The interpretation that they contain polymerized DNA rests on the finding of KURNICK (**8**) that methyl-green stainability of solutions of DNA depends on their state of polymerization.

Cytochemical evidence leading to the same conclusions was presented by POLLISTER and LEUCHTENBERGER (**13**) in studies of epithelial cells of mouse liver. Cytoplasmic inclusions of DNA similar to those reported here have been observed in root tips of onions treated for more than 24 hours with suspensions or filtrates of "Spergon" (**16**). These findings suggest that DNA escapes from the nuclei; but whether this material is removed as a high polymer, or as a low polymer which is then repolymerized in the cytoplasm, is not known.

KURNICK has also shown that depolymerized DNA has a greater affinity for pyronin than has polymerized DNA. In conformity with this interpretation it may be assumed that the intense violet color of the chromatin of pycnotic cells (as contrasted with the blue of nonpycnotic cells) results from depolymerization of DNA, with a decrease in the methyl green–staining component and a corresponding increase in the pyronin-staining component. This assumption is supported by the observation that stainability of the chromatin in these cells is markedly reduced by desoxyribonuclease but not by ribonuclease or pepsin when used separately or in combination. LEUCHTENBERGER (**9**) also has reported that pycnosis involves depolymerization of DNA.

If one adopts the interpretation of KURNICK concerning stainability with pyronin, it might be assumed that the perinuclear spots represent depolymerized DNA. But this assumption is not sustained by the facts that they are Feulgen-negative and that their stainability is not impaired by the action of desoxyribonuclease. Another possibility is that the perinuclear spots are composed of ribose nucleic acid (RNA), which is also known to stain with pyronin. Treatment with ribonuclease partially reduced but did not eliminate stainability of the spots with pyronin. This suggests that the perinuclear spots contain some RNA. Pepsin alone also partially reduced pyronin stainability of these spots. When treatment with ribonuclease was followed or preceded by treatment with pepsin, colorability was completely eliminated. From these observations it may be concluded that the perinuclear spots contain protein as well as RNA.

The staining reactions of the nucleoli in the pycnotic cells and their response to treatment with pepsin and ribonuclease closely paralleled the behavior of the perinuclear spots. It may be assumed by analogy that they are constituted of similar materials. The pattern of organization of these materials differs, however, from that in the nonpycnotic cells, in which pyronin stainability of nucleoli was eliminated by treatment with ribonuclease alone. There is also some evidence that these materials do not represent merely a re-association of those existing in the nucleoli before chemical treatment, since the size and number of these bodies were increased in the course of pycnosis. This suggests that translocation of materials is involved, perhaps by transfer to the nucleoli and to the perinuclear pools from other parts of the nuclei, which became smaller during the course of pycnosis. The precise source, however, remains to be determined.

Summary

1. The effects of colchicine, caffeine, hydroquinone, pyrogallol, pyrocatechol, 8-hydroxyquinoline, and *p*-aminodimethylaniline monohydrochloride on root-tip cells of *Allium cepa* and *Lilium tigrinum* were determined by cytological observations and cytochemical procedures, em-

ploying purified ribonuclease, desoxyribonuclease, and pepsin.

2. The morphological changes induced by the chemicals included: c-tumor formation (by colchicine); c-mitosis (by colchicine, hydroquinone, and pyrogallol); chromosome fragmentation (by caffeine, hydroquinone, and pyrogallol); failure of cell-plate formation (following treatment with caffeine); increase in nucleolar volume (in cells of all chemically treated roots); and pycnosis with characteristic agglutination of chromatin (produced by all chemicals except caffeine under suitable conditions).

3. No difference was found between the tissues of chemically treated and untreated roots with respect to stainability or digestibility of their nucleic acids and proteins with purified enzymes, unless the chemicals caused pycnosis.

4. In some pycnotic cells the over-all basophilia of cytoplasm and nucleus was reduced, and filaments containing desoxyribonucleic acid (DNA) were observed in the cytoplasm. Cytochemical analysis indicated that the DNA was polymerized. In general, however, pycnotic nuclei were characterized by shrinkage and intense, uniform staining, with loss of individuality of chromosomes. When stained with pyronin–methyl green, the chromatin of these cells appeared violet, and there were pools of deep-red-staining material in the nucleoli, around the nuclei, and around the condensed chromosomes. Cytochemical analysis suggested that these red-staining materials consisted of both ribonucleic acid and protein. The analysis also indicated that the violet color of the nuclei was probably due to the presence in agglutinated chromatin of both polymerized and depolymerized DNA.

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DEPARTMENT OF GENETICS
CARNEGIE INSTITUTION OF WASHINGTON
COLD SPRING HARBOR, NEW YORK

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THE ROLE OF COUMARIN IN THE GROWTH OF ROOTS OF MELILOTUS ALBA[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY **635**

JAMES P. SAN ANTONIO

Introduction

Among the different kinds of substances shown to inhibit various plant processes, those classified as aglycones are of special interest. They or their glycosides (in combination with a sugar) have been isolated from many different orders of plants (**1**). Though they occur naturally, relatively little is known regarding their role in plant metabolism. ARMSTRONG and ARMSTRONG (**1**) considered that they may function variably depending on the particular plant.

One important class of aglycone compounds is that containing the structural grouping shown in figure 1. Of these unsaturated lactones, the substance coumarin (fig. 2) and closely related compounds (figs. 3, 4) are widely distributed in plants. WEHMER (**26**) listed more than fifty different plants as sources of coumarin. SETNA and SHAH (**20**) stated that about fifty natural derivatives of coumarin had been isolated up to 1945.

Coumarin elicits differing responses in a variety of plants. These have been reviewed in the literature and may be classified as: (*a*) inhibition of seed germination (**3, 25**), of mitosis (**7, 10**), of root growth (**2, 10**), and of cell elongation in stems (**23, 24**) and (*b*) stimulation of hypocotyledonary thickening and lateral root formation (**2**) and of expansion of laminar leaf tissue (**16**).

The process inhibited in root growth has not been definitely established, but that unsaturated lactones may interfere with mitosis is evident in the literature cited by GOODWIN and TAVES (**10**). The extent to which they may inhibit cell elongation in roots has not yet been ascertained.

Relatively few studies have been made of the effects of coumarin and its derivatives on plants which normally contain such compounds. GOODWIN and TAVES (**10**) have shown that scopoletin (6-methoxy-7-hydroxy coumarin) inhibits root growth of *Avena* seedlings. The concentration of scopoletin in *Avena* root tissue has been determined by GOODWIN and KAVANAGH (**9**). BRINK (**5**) surveyed the effects of a number of coumarin derivatives on the root growth of sweetclover and found that coumarin and

[1] This work was supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

melilotic acid inhibited root growth of sweetclover and alfalfa seedlings. o-Coumaric acid did not inhibit sweetclover root growth. Leaves, stems, and seeds of sweetclover have long been known to contain coumarin and other closely related compounds (**25**).

Attempts have been made to correlate the activity displayed by many unsaturated lactone compounds with their chemical structure. The extensive literature on this subject indicates that the inhibitory activity is associated with the characteristic molecular grouping (fig. 1) (**10, 24**).

CAVALITTO and HASKELL (**6**) showed that unsaturated lactones of the Δ,β,γ angelicalactone type combined with cysteine in vitro. Coumarin, however, did not react with cysteine. The addition of 1,2-dimercaptopropane, generally considered to "protect" sulfhydryl groups, to a solution containing coumarin, has been shown by THIMANN and BONNER (**23**) to decrease greatly the inhibitory effect of coumarin on the growth of *Avena* coleoptile sections.

The marked inhibitory effects of coumarin and its derivatives suggested that experiments on the role of coumarin in the growth of a plant naturally containing it might yield information regarding the function of this type of substance. Thus, the influence of coumarin and certain of its derivatives on the growth of roots of sweetclover, which contains such substances, was studied.

The experiments had several objectives: (*a*) to test the effects of coumarin, o-coumaric acid, and melilotic acid on the root growth of sweetclover seedlings and on cell elongation; (*b*) to establish the possible significance of the structural differences of these compounds; (*c*) to ascertain any correlation between growth and the distribution of these substances within the root; and (*d*) to determine the occurrence of these substances during very early stages of the germination of sweetclover seeds.

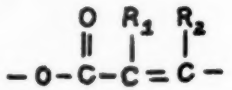


FIG. 1

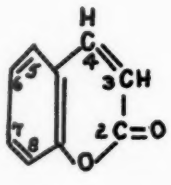


FIG. 2.—Coumarin

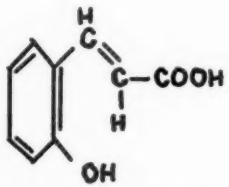


FIG. 3.—o-Coumaric acid

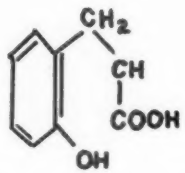


FIG. 4.—Melilotic acid

Material and methods

In order to facilitate quantitative determinations of coumarin in small samples of tissue, two pure strains of sweetclover (*Melilotus alba* Desr.), Evergreen sweetclover (FC 23 811)[2] and Com-

[2] Provided through the courtesy of Professor W. K. Smith, Wisconsin Agricultural Experiment Station, Madison, Wisconsin.

mon Biennial White sweetclover (FC 23 183),[3] both with a high content of coumarin, were selected for the experiments. For various comparisons and control experiments a related plant species containing no coumarin was used—alfalfa (*Medicago sativa* L.), variety Colorado (5068 C).

Synthetic coumarin was obtained from Eastman Kodak Company. This preparation, reprecipitated from hot petroleum ether, had a melting point of 69.5°–69.75° C. o-Coumaric acid was prepared according to the method of REYCHLER (**17**). Melilotic acid was prepared by saponification and subsequent purification of the lactone of melilotic acid which was obtained in a fused state from Fries Brothers, New Jersey. The melilotic acid, after repeated recrystallizations from hot carbon tetrachloride, had a melting point of 88.0°–88.5° C.

The diethyl ether used in all quantitative procedures was Mallinckrodt no. 0848, which before use was distilled from an alkaline reducing mixture and dried by freezing.

Determinations of amounts of coumarin and melilotic acid were made by a colorimetric method. Coumarin was also determined fluorometrically.

The colorimetric determination was adapted from ROBERTS and LINK (**19**) and BRINK (**5**), who measured the color intensity of the compound formed by the coupling of coumerinic acid (the cis form of o-coumaric acid) with diazotized p-nitraniline. The procedure used in these experiments was as follows:

(*a*) Aqueous test solutions, measuring 1–5 ml. and containing more than 10 but less than 50 μgm. of coumarin, were pipetted into Pyrex test tubes graduated for 10 ml.

(*b*) To each tube was added 1 ml. of a Na_2CO_3-H_2SO_4 solution (20 gm. of Na_2CO_3 plus 27 ml. of 0.9 *N* H_2SO_4 in 2 l. of distilled water, with a pH of 10.2, as measured with a Beckman glass-electrode pH meter).

(*c*) Volumes of all solutions were brought to 6 ml. by addition of distilled water, where necessary, and mixed.

(*d*) Tubes containing the unknown concentrations and those containing a series of known concentrations of coumarin were then heated in a water bath at 85° C. for 20 minutes.

(*e*) The test tubes were placed in a temperature-controlled water bath at 25° ± 0.5° C.

(*f*) After 10 minutes the first standard-solution test tube was removed from the bath, 1 ml. of a diazonium salt solution was added from a burette (**19**), and the solution was quickly made up to volume with the buffer described in (*b*). The test tube was inverted twice to insure homogeneity of the cherry-colored azo dye formed by the reaction.

(*g*) Color intensity was measured with the Klett-Summerson photoelectric colorimeter equipped with a test-tube adaptor. The filter KS-52 with transmission limits of 485–550 μ was used. The colorimeter scale reading was directly proportional to the concentration of coumarin. A number of control determinations were performed along with each group of unknowns.

Determination of melilotic acid concentration followed the above procedure through step (*e*). After the 10-minute cooling period, 0.5 ml. of 0.5 *N* H_2SO_4 was added to the test tube, and the contents were mixed. The acidified solution was returned to the bath for at least 15 minutes, then 1 ml. of the diazonium so-

[3] Provided through the courtesy of Dr. A. E. Hollowell, Division of Forage Crops and Diseases, United States Department of Agriculture, Beltsville, Maryland.

lution was added, and the test tube was inverted twice. Then 0.5 ml. of 0.5 *N* NaOH was added, and the volume was quickly made up to 10 ml. with the buffer solution (*b*). After mixing, the solution was measured in the colorimeter. As with the determination of coumarin concentration, parallel determinations of known concentrations of melilotic acid were made in addition to unknowns. In this procedure the standard curve was usually similar to that for coumarin but somewhat less consistent from day to day. Melilotic acid is measured by the same procedure as coumarin, whereas coumarin does not yield a color in the melilotic acid procedure. Thus, specific determination of either substance requires both procedures described and the appropriate subtraction when calculating the results.

Determinations of either coumarin, melilotic acid, or mixtures of these substances on separate aliquots were reproducible within 3%.

The quantitative fluorometric determination of coumarin was adapted from SLATENSEK and WASHBURN (**22**) and SLATENSEK (**21**). The method employed here, mainly in the analysis of root extracts, however, did not include the use of an alfalfa extract as an ever present blank since the quantity of extraneous fluorescent material in alfalfa seedling roots was negligible.

The steps in the procedure were:

(*a*) The aqueous solutions to be tested, measuring 1–9 ml. and containing 1–10 μgm. of coumarin, were pipetted into test tubes graduated at 10 ml.

(*b*) The hydroxyl-ion concentration of these solutions was adjusted to 0.1 *N* by addition of NaOH solution.

(*c*) After mixing, the solutions were heated for 15 minutes at 85° C.

(*d*) After cooling, the tubes were placed so that the solutions were exposed 10 minutes either to direct sunlight or to light from an artificial ultraviolet source. The solutions, then at maximum fluorescence, were poured into test tubes fitting the fluorometer, and their fluorescence was measured with the Coleman photofluorometer No. 12. Filters B-1 and PC-1 were employed. Sensitivity of the instrument was kept constant by use of a reference solution of quinine sulfate (**12**:316).

It was necessary to establish a standard curve each time a series of solutions of unknown concentrations was measured, since scale readings were not always proportional to concentration. As a safeguard, a standard curve based on seven to ten determinations was always established both before and after measurement of unknown solutions. This procedure permitted duplicate determinations of an unknown concentration within ±5%. All quantitative determinations of substances reported in the various experiments are averages of at least two analyses.

Experimentation and results

EFFECT OF COUMARIN, O-COUMARIC ACID, AND MELILOTIC ACID IN AVENA CURVATURE TEST

PROCEDURE.—This test (**14**) was used to determine the effects of these substances on cell elongation. The procedure consisted of introducing, by the ether-dropping method (**4, 13**), known amounts of indoleacetic acid and one of the inhibitors into an agar square of 0.1-ml. volume. This square, after at least an hour, was divided into blocks of 0.04-ml. volume. These were affixed unilaterally to doubly decapitated *Avena* coleoptiles growing in a dark room maintained at 60% relative humidity with a temperature of 22°–23° C. Operations were per-

formed in red light (Corning light filter no. 243). The agar contained 1.25% of thoroughly washed agar, 0.005 *M* Sorensen's citrate buffer, and 0.001 *M* calcium chloride, and the pH was 6.0.

Concurrent with the application of mixtures of indoleacetic acid and inhibitor, a control experiment was performed in which a known quantity of indoleacetic acid only was applied. The indoleacetic-acid concentration used in any given mixture was that which alone yielded a curvature of 15°, 30°, or 50°. Each point on the final curve represents an average of at least two *Avena* tests.

RESULTS.—The magnitude of coleoptile curvatures resulting from application of various mixtures of indoleacetic acid and inhibitor compared with curvatures obtained with indoleacetic acid alone gave an expression of activity of the inhibitor (fig. 5). Coumarin had no activity in this test, despite the fact that THIMANN and BONNER (23) have demonstrated that coumarin inhibits growth in the *Avena* coleoptile section test. This activity in the present test was probably a result of failure of coumarin either to enter or to be transported in the coleoptile, rather than because it failed to act as an inhibitor. This was suggested by the activity of o-coumaric acid in this same test. Further, the inhibitor effect of o-coumaric acid was of the same magnitude reported by THIMANN and BONNER for coumarin in the coleoptile section test.

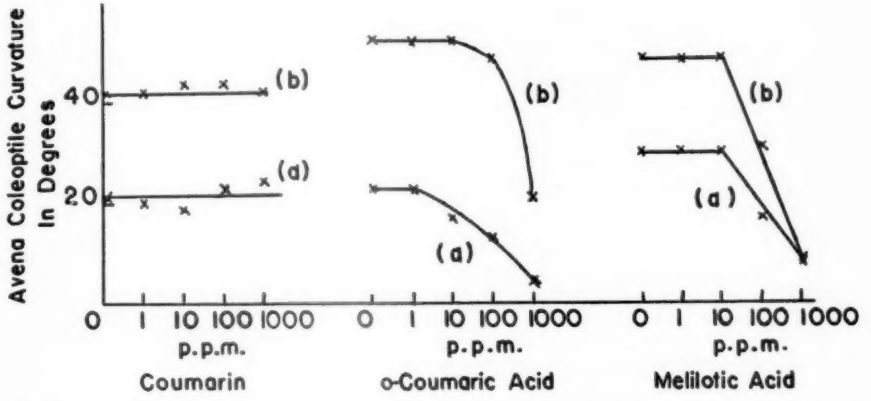


FIG. 5.—Effect of mixtures of indoleacetic acid and coumarin or related substances on *Avena* coleoptile curvatures. All (*a*) curves represent curvatures yielded by mixtures of 30 μgm/l of indoleacetic acid and various concentrations of the given inhibitor. Results shown in (*b*) curves are of *Avena* tests in which concentration of indoleacetic acid in the various mixtures was 120 μgm/l. An inhibitor concentration of 0 p.p.m. indicates that indoleacetic acid alone was applied (control).

The inhibitory effect of melilotic acid was evinced at a lower concentration than that of o-coumaric acid. This, however, may be an effect of hydrogen-ion concentration affecting entrance and transport, rather than a true difference in inhibitory activity.

EFFECT OF COUMARIN AND COUMARIN DERIVATIVES ON GROWTH OF SWEETCLOVER AND ALFALFA ROOTS

The variety Common Biennial White sweetclover was used for all experiments reported on root growth. Experiments with roots of alfalfa seedlings were per-

formed parallel with those on sweetclover roots. This provided a comparison of the inhibitory effect of these substances on sweetclover, containing coumarin compounds, with a related plant which contains only melilotic acid (**19**).

GENERAL EQUIPMENT.—Cardboard boxes, 1×25×30 cm., with the inside of the covers and bottoms covered with paraffin, proved satisfactory as germination chambers. Seeds were laid in four rows of about thirty seeds each on a layer of blotting paper wet with distilled water.

For later growth an agar substrate at pH 6.0, containing 1.25% of thoroughly washed agar and 0.005 *M* Sorensen's citrate buffer, was used. Various concentrations of the inhibitors tested were made up in 20-ml. volumes of this agar in Petri dishes 7 cm. in diameter. Several hours after solidification each agar disk was cut along the diameter, and one-half was removed to a second Petri dish. Thus, each dish contained a half-circle of agar of 10-ml. volume. Seedlings were placed on the agar half-circle so that the uppermost root hairs coincided with the cut upper edge of the agar (fig. 8). Except at transfer and measurement of the plants, the containers were placed so that the substrates made a 75° angle with the horizontal, with the roots pointing downward.

From the beginning of germination onward, all operations and measurements were carried out in the dark room in red light at 60% relative humidity and at a temperature of 22°–23° C.

PROCEDURE.—About 1,500 seeds of sweetclover or alfalfa were soaked in 2 l. of distilled water for 15–18 hours. The water was decanted, and the seeds were rinsed with at least three volumes of distilled water to remove leached coumarin. Seeds were then transferred to the blotting paper and oriented so that the radicle would grow straight downward. The boxes were covered and placed in the dark room.

In 24–36 hours, germinated seedlings having straight roots 10–15 mm. long were transferred to Petri dishes with agar containing no coumarin. After growing 24 hours on agar, all seedlings that did not have straight roots 18–25 mm. long were discarded. On each remaining root, 5–6 mm. behind the root tip, a small mark of ink (carbon black mixed with paraffin oil) was placed with a celluloid nib. The time was noted, and the distance between the mark and the root tip measured, using a binocular dissecting microscope with an ocular micrometer at 5.25×. Measurements were recorded consecutively in sets of ten.

After 2 hours, ten plants, in consecutive order, were transferred to a Petri dish containing agar with a known concentration of coumarin. When they were in place, the time was noted, and the distance between mark and root tip was measured. The growth increment calculated from these two sets of measurements was taken as the pre-experimental growth increment for a given set of roots. The time required for transfer and measurement did not exceed 5 minutes. Subsequent measurements were made at noted time intervals. In control experiments similarly measured and transferred roots were placed on agar containing no coumarin.

RESULTS.—Experiments with a concentration of 1 p.p.m. of coumarin were inconclusive and are not reported. The total length increment of roots of sweetclover and alfalfa seedlings grown on agar containing 5 p.p.m. of coumarin was about 55% and 40%, respectively, of the control (figs. 6, 7). At a concentration of 50 p.p.m. of coumarin, the increment of

sweetclover roots was further decreased by about 10%; alfalfa roots, however, did not increase in length at all after they were placed on such agar.

Melilotic and o-coumaric acids also inhibited growth of sweetclover and alfalfa roots (table 1). Sweetclover roots were not inhibited as markedly by melilotic acid as were alfalfa roots.

Both sweetclover (fig. 9) and alfalfa roots grown on agar containing 5 p.p.m. of coumarin exhibited pronounced subterminal root swelling. Application of 50 p.p.m. of coumarin did not elicit such marked radial increase.

Determination of growth zones of sweetclover and alfalfa roots

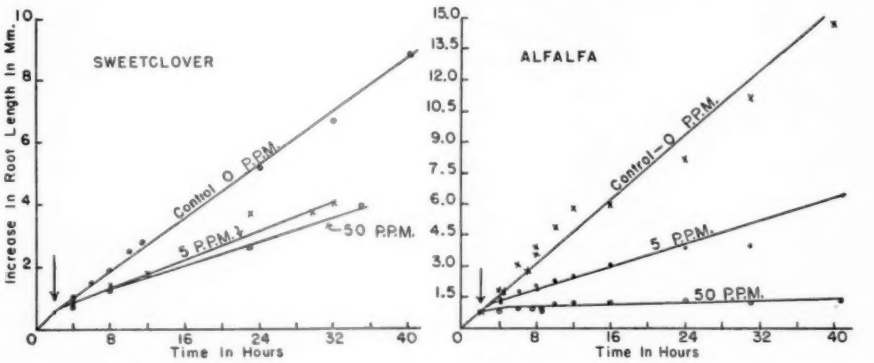
Common Biennial White sweetclover seedlings were germinated and grown in the manner described for the study of the effect of coumarin inhibition. Only the first transfer to agar was required, however.

Selected roots, 18–25 mm. long, were marked with ink at 1-mm. intervals for a distance of 5 mm. starting from the root tip. Each marked zone of a given root was then measured, using an ocular micrometer at 30× magnification, and the time was noted. A second measurement of these zones of the same root was made 2 hours later.

The average growth increments of samples consisting of ten roots were similar, but, as would be expected, the increment of any one root within a sample varied from the average. For this reason it was more accurate to compute the length increase of a particular zone on the basis of the total length increase for a given root rather than on the basis of the average length increase of the sample. Especially is this true of relatively slow-growing roots and of roots in which the length of the growing zone is short, as with these roots.

In calculating the growth for zone x of a given root: A = total increment; B = increment of zone x; (100 B)/A = % of the total increment occurring in zone x.

Ten such calculated percentages for each of the five zones measured were



Figs. 6, 7.—Effect of 5 and 50 p.p.m. of coumarin on growth of roots of (fig. 6, *left*) sweetclover (var. Common Biennial White) and alfalfa (fig. 7, *right*). Arrow indicates time at which roots were transferred from water agar to either control agar or agar containing coumarin. Calculation of results involved correction for varying initial growth rates during 2-hour pre-experimental growth period. Each point represents average of increments of three to six separate samples. Same roots were measured at different times.

averaged. Each point in figure 10 represents a further average of at least three such samples. From figure 10 it is apparent that almost 75% of the total root growth occurred in the second millimeter from the root tip. Cell elongation more than 3 mm. from the tip was negligible. The distribution of growth in alfalfa roots was similar to that in sweetclover (fig. 10).

Longitudinal distribution of coumarin in sweetclover roots

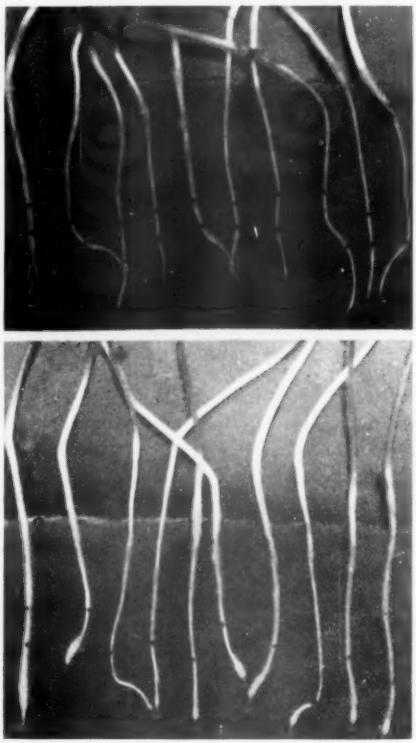
Two forms of coumarin, free and bound, occur in sweetclover tissue (**19**). Depending on the method of extraction, a given weight of tissue may yield two different quantities of coumarin. Free coumarin is the quantity extractable with organic solvents from freshly ground tissue. In tissue which has undergone an incubation period or has been heated with acid or base, the hydrolytic treatment serves to liberate the so-called "bound" coumarin from glycosides or other combined forms. Thus, organic solvent extraction of incubated or hydrolyzed tissue removes the bound coumarin fraction in addition to the free coumarin fraction. The following relationship then holds: total coumarin − free coumarin = bound coumarin.

For study of the distribution of coumarin, about two hundred Common Biennial White sweetclover seedlings were removed from agar and lined up on dampened blotting paper.

For extraction of free coumarin, seedlings with roots about 25 mm. long were taken to a cold room, 0° C., and the roots were cut with a razor blade into 1-, 2.5-, or 5-mm. sections. The tissue was quickly blotted with filter paper and weighed to the nearest 0.2 mg. on a torsion balance. The tissue weight, depending on the number of roots and the location of the zone, ranged from 10 to 250 mg. A zone was designated by its distance from the root tip.

The tissue was then transferred to a small test-tube mortar containing a few grains of washed sand. Chilled 95% ethyl alcohol, 0.5 ml/100 mg of tissue, was added, and the tissue was thoroughly ground with a glass pestle. After the tissue had been reduced to a powder-like fineness, about 5 ml. of peroxide-free ether was added, and the tissue was again ground.

The following operations were made at room temperature. The solvent was de-



FIGS. 8, 9.—Fig. 8 (*above*), sweetclover roots grown for 24 hours on control agar. Note ink marks used in measurement. Fig. 9 (*below*), sweetclover roots grown for 24 hours on agar containing 5 p.p.m. of coumarin. Note subterminal swellings of root tips.

canted through a sintered glass filter of medium fineness under suction into a 50-ml. Erlenmeyer flask. The tissue was again ground in 95% alcohol (0.5 ml. total), then 3 ml. of ether were added, and the grinding was repeated. The solvent was then decanted through the filter. This procedure was repeated once more, to yield about 12 ml. of an alcohol-ether extract containing the free coumarin.

Roots from which total coumarin was to be extracted were cut and weighed in the same manner as in the free coumarin extraction method, except that the operations were performed at room temperature. After addition of 0.5 ml. of distilled water, the tissue was thoroughly ground. The mortars, corked, were then placed in an incubator at 40° C., and the tissue was incubated for 1 hour, then removed from the incubator and extracted with alcohol and ether as described for the extraction of free coumarin. First, however, as much of the aqueous supernatant as possible was decanted through the filter. There then followed three alcohol-ether extractions.

From this point on, both kinds of extracts were treated alike. Fifteen milliliters of distilled water were added to each flask containing the extracts. The ether was then carefully evaporated at 45° C., using a gentle stream of com-

TABLE 1

GROWTH, AS PERCENTAGE OF TOTAL INCREMENT OF CONTROL, OF ROOTS OF SWEETCLOVER* AND ALFALFA SEEDLINGS IN 4 AND 24 HOURS† ON AGAR CONTAINING EITHER o-COUMARIC ACID OR MELILOTIC ACID

Concentration of inhibitor (p.p.m.)	Sweetclover		Alfalfa	
	4 hours	24 hours	4 hours	24 hours
	o-Coumaric acid			
5	82.0 74.4 (86.0)‡ 104.0	102.0 144.0 (119.0) 112.0	88.5 92.0 (90.2)	93.6 118.0 (105.8)
50	66.4 52.1 (59.2)	99.3 114.0 (106.0)	62.3 47.4 (54.8)	56.5 48.2 (52.3)
100	5.8	0.0	36.3 9.5	26.3 0.0
250	9.8 5.8 (7.8)	0.0	.....	.....
500	0.0	0.0	0.0	0.0
	Melilotic acid			
5	113.0 (118.0) 133.0 109.0	123.0 153.0 (128.0) 109.0	18.6 0.0	10.6 0.0
50	86.9 28.8	109.0 64.6	17.6 0.0	3.8 0.0

* Var. Common Biennial White sweetclover.

† Same roots were measured at 4 and 24 hours.

‡ Figures in parentheses are arithmetic means of different sample percentages with which they are associated.

pressed air. To each flask was then added 0.75 m.e. of H_2SO_4, and the solutions were heated at 70° C. for 15 minutes to destroy interfering pigments. After cooling, the contents of each flask were filtered through a sintered glass filter of

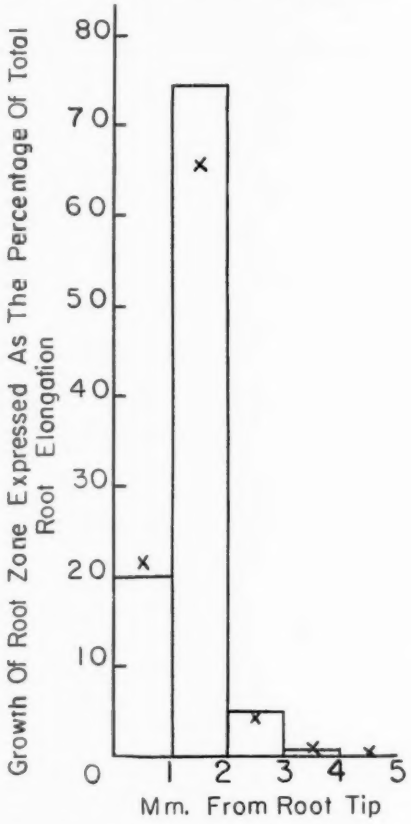


FIG. 10.—Longitudinal distribution of growth in roots of Common Biennial White sweetclover (histogram) and alfalfa (points at *x*) seedlings.

medium fineness under suction into a 10- or 25-ml. volumetric flask. The Erlenmeyer flasks were rinsed two times with 3-ml. portions of distilled water. The solutions were then made to volume and mixed. Aliquots of these aqueous solutions were then prepared for either fluorometric or colorimetric analysis of coumarin content as described earlier. Various control experiments were performed to test the procedure described (see below).

RESULTS.—The determinations of free and total coumarin in specific root zones are presented in table 2. The wide range

TABLE 2

OCCURRENCE OF COUMARIN IN ROOTS OF COMMON BIENNIAL WHITE SWEETCLOVER EXPRESSED AS μGM COUMARIN/GM FRESH WEIGHT

LOCATION* AND LENGTH OF ZONE	μGM OF COUMARIN†	
	Extracted as free coumarin	Extracted as total coumarin
1st mm.	61.5	.....
2d mm.	70.3	.....
3d mm.	43.0	.....
4th mm.	45.6	.....
5th mm.	97.8	.....
1st 2.5 mm.	118.0 (120.0)	218.0 (187.0)
	74.8	144.0
2d 2.5 mm.	76.5 (62.3)	256.0 (266.0)
	85.8	289.0
2d 5 mm.	78.2 (72.8)	154.0
3d 5 mm.	77.4	222.0
4th 5 mm.	69.8	.....

* Zone is designated by distance from root tip.

† Majority of figures given are those based on fluorometric analyses. Figures included in parentheses, however, represent duplicate analyses made calorimetrically.

of values reported may result from several causes: (*a*) the inherent variability of the root tissue, and (*b*), though recovery tests yielded satisfactory results, the extraction of tissue involves factors which are not so readily tested. Among these are the differences in fineness to which tissue may be ground and the speed with which the solvent enters the tissue. In addition, it is possible that some hydrolysis of bound coumarin could have occurred in a varying degree despite the conditions employed to minimize this.

It is evident (table 2) that in the tip 3 mm. of the root the concentration of

coumarin is as high as that elsewhere in the root. The average concentration of free coumarin is at least 74 μgm/gm fresh weight. Determinations of the concentration of free o-coumaric acid according to the method of ROBERTS and LINK (**18**) indicated that only 20% of the values found for coumarin resulted from the presence of coumaric acid. In view of the difficulties in measuring minute quantities of coumaric acid, results have been presented in terms of coumarin. Colorimetric measurements indicated that melilotic acid was present only in negligible quantities. Further, fluorometric analysis, used for the major part in these experiments, does not measure melilotic acid. There was substantial agreement (table 2), in limited duplication by colorimetric analysis, with the results yielded by fluorometry.

The blank value of alfalfa tissue was a negligible factor. The content of coumarin-like substances in roots of alfalfa seedlings expressed as micrograms of coumarin per gram of fresh weight was 4.30, 3.30, 3.86, 8.22, and 5.32 for the first millimeter from the root tip to the fifth millimeter, respectively. In this control experiment the tissue was treated as for extraction of free coumarin, and the solution for analysis was measured fluorometrically. In addition, two control experiments were performed in which coumarin was added to alfalfa root tissue, and the tissue was then extracted for free coumarin. Of the added coumarin, 93.9% and 97.0% were recovered.

LEACHING OF COUMARIN FROM SEEDS

Preliminary experiments indicated that an appreciable quantity of coumarin was present in water in which sweetclover seeds had been soaked. Since coumarin inhibits germination of various species, a quantitative measure was desirable of the extent to which coumarin leached out of the seeds. MCCALLA and DULEY (**15**) presented data indicating that coumarin was leached from sweetclover hay soaked in water. The derivation of an inhibitory substance (coumarin) from sweetclover tissue is not included by EVENARI (**8**) in his large list of inhibitor sources.

SEED IMBIBITION.—In experiments reported here, only "selected" sweetclover seeds were used. These were chosen for both uniform olive-green seedcoat color and for seeds without chipped or cracked surfaces. A set of one hundred selected seeds weighed 0.200 gm. ± 2.5%. Evergreen sweetclover seeds were used in all experiments except one (table 3).

TABLE 3

TOTAL μGM OF COUMARIN AND RELATED SUBSTANCES LEACHED FROM 100 IMBIBING SWEETCLOVER SEEDS (VAR. COMMON BIENNIAL WHITE FC 23 183)

Time soaked (hrs.)	Total coumarin (μgm.)
1.5	464.0
2.0	396.0
2.0	363.0
2.0	343.0
3.5	403.0
3.5	477.0
6.0	643.0
6.0	511.0
8.0	555.0
8.0	602.0

Determination was made of amount of water imbibed by sweetclover seeds during soaking. Seeds (0.2 gm.) were soaked in distilled water and at intervals from zero time were removed, rapidly blotted on filter paper, weighed to the nearest milligram, and then returned to the water. After 90 minutes the hard seeds were removed, dried, weighed, and discarded. In calculating imbibition data the weight of the hard seeds was subtracted from the initial dry weight and

the successive wet weights. In addition, (*a*) six sets, of one hundred seeds each, were soaked in separate jars containing 10 ml. of a 100-p.p.m. coumarin solution, and (*b*) another six sets were soaked in distilled water. Different sets were soaked for intervals of time ranging from 0 to 320 minutes. Each set of seeds was weighed only once and then discarded. Thus, a time series of imbibition derived from twelve different sets of seeds, with and without coumarin, could be constructed. A correction for the weight of hard seeds was made in each case.

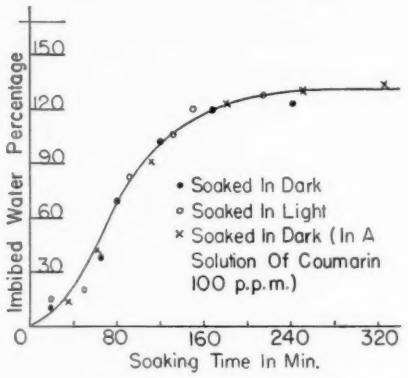


FIG. 11.—Imbibition of water by sweetclover seeds (var. Evergreen). Quantity of water imbibed is expressed as percentage of initial air-dry weight of seeds.

Figure 11 indicates that imbibition was completed after 200 minutes; the seeds had then imbibed a weight of water equal to 130% of their initial dry weight. It is also apparent that neither the rate nor the magnitude of imbibition was affected by the presence of coumarin in the solution in which the seeds were soaked.

COUMARIN DETERMINATIONS.—Sets of one hundred sweetclover seeds were soaked for varying lengths of time, under controlled conditions, in 5 ml. of distilled water. After a given time the liquid was carefully decanted into a 10-ml. volumetric flask. The seeds were rinsed twice with 2 ml. of distilled water, and the rinse water was also decanted, after each rinsing, into the flask. After making up the volume to 10 ml. and mixing, an aliquot of 9 ml. was pipetted into a 45-ml. separatory funnel. The solution was adjusted to pH 1.5 by addition of 0.4 ml. of 1 *N* HCL, then shaken 4 minutes with 9 ml. of peroxide-free ether, and the colorless ether fraction was collected in a 25-ml. flask. The aqueous solution was then similarly shaken with fresh ether twice more, and the ether fractions were pooled. After the ether solution was made up to a volume of 25 ml., the entire fraction was evaporated at 45° C. in a flask containing 15 ml. of distilled water. The coumarin content of aliquots of the resulting aqueous solution (first made up to a volume of 25 ml.) was measured in the manner described earlier except that this final aqueous solution was not heated with acid as in the root-extraction experiments.

Surface sterilization of seeds (after treatment with 1% bromine solution for 1 minute, seeds were drained for 1 hour on sterile filter paper) in conjunction with sterile technique throughout the experiment did not significantly affect the magnitude of the substances assayed. Thus, for the later experiments, seeds were not treated with bromine. No distinction is made regarding this difference in data presented.

For those experiments in which seeds were soaked in the light, a bank of fluorescent tubes yielding an incident light intensity of 300 foot-candles was used. For experiments in which seeds were soaked in the dark, they were prepared in red light (Corning filter no. 243) and thereafter during the soaking period exposed to only occasional red light. A temperature of 22°–23° C. was main-

tained in both light and dark experiments.

In three separate control experiments in which aqueous solutions containing known amounts of coumarin were shaken with ether at pH 1.5, 103%, 100%, and 102% of the coumarin was recovered.

The germination percentage of seeds soaked in these experiments was determined in most cases 72 hours after the end of any soaking period. The mean of seventeen determinations (72–84% germination) was 79.5%, which did not differ significantly from the percentage of imbibed seeds. Thus, from the germination percentage it was possible to correct for the presence of hard seeds and so calculate the quantity of leached substances on the basis of one hundred imbibing seeds.

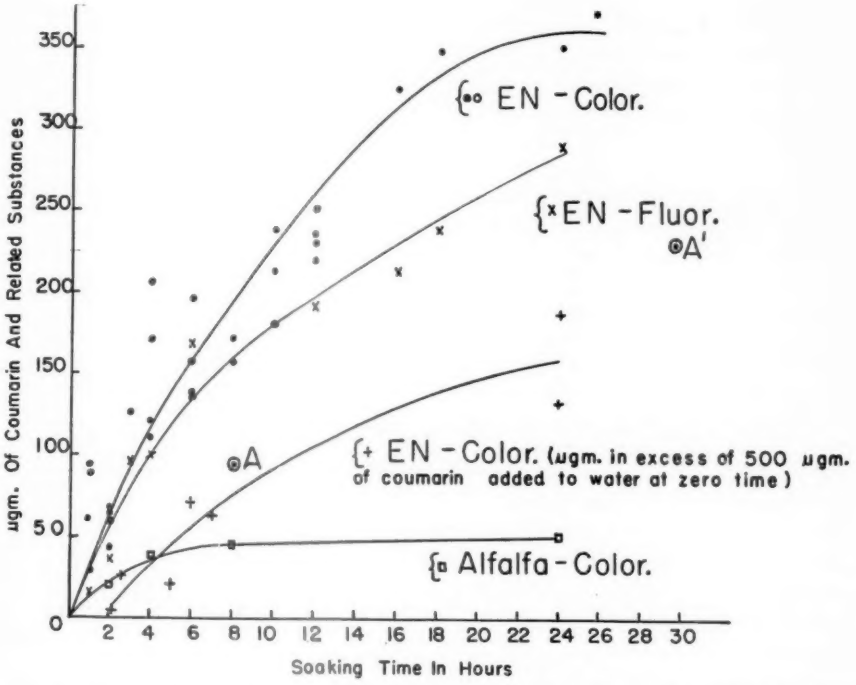


FIG. 12.—Determination of coumarin and related substances in 5 ml. of water in which seeds of sweetclover (var. Evergreen, abbreviated EN in figure) or alfalfa were soaked for varying lengths of time in light or in dark at 22°–23° C. Abbreviations *Color.* and *Fluor.* signify method by which solutions were analyzed, colorimetrically and fluorometrically, respectively. Open circles and squares indicate that seeds were soaked in light; and all other points of experiments in which seeds were soaked in dark. Points *A* and *A'* represent results of experiments in which leachate solutions were shaken with ether at pH 10.2.

RESULTS.—The total quantity of substances leached per one hundred imbibing seeds (var. Evergreen) for different lengths of time soaked is given in figure 12. The substances leached were contained in 5 ml. The error introduced by neglecting the quantity of water imbibed by the seeds amounts to only about 6%. It is evident that for the first 24 hours of soaking the total amount of coumarin

and related substances leaching out of the seeds increased with the length of time the seeds were soaked. It is also evident that there was no substantial difference between the quantity of substances leached from seeds soaked in the light and from those soaked in the dark.

Prior to 20 hours the radicle became progressively more defined. After this time the seed became extremely soft, and the radicle exhibited geotropic curvatures.

The variability of results could have resulted in part from the presence of chipped, cracked, or off-color seeds which, despite careful culling, escaped detection. To determine the effect of the seedcoat on the rate with which coumarin was leached, seedcoats of seeds (var. Evergreen) were nicked with a razor blade before soaking in 5 ml. of water. The total μgm. of coumarin and related substances leached from one hundred nicked imbibing seeds was 213.0, 225.0, 235.0, and 253.0 for 2, 4, 8, and 8 hours soaking time, respectively. The total μgm. of coumarin and related substances leached from another set of seeds, not nicked with a razor blade but composed, however, of seeds having either cracked or off-color seedcoats, were 636.0 at the end of a 24-hour soaking period. Limited data (table 3) indicate that the amount of substances leaching out may also vary according to the strain of the seed.

Data of recovery tests (fig. 12) indicate that the presence of a high initial concentration of coumarin (100 p.p.m.) in the soaking water decreased the quantity of coumarin leaching out of the seeds. As an indication that the greater portion of the substances assayed consisted of coumarin—a neutral substance—the results of two control experiments, in which the solution containing the leached substances was shaken with ether at a pH of 10.2, are included in figure 12. The smaller amount of leached substances is attributed to the failure of coumaric and melilotic acids to pass from water to ether at this pH.

On the basis of the fluorometric analysis (which may measure fewer extraneous substances, such as melilotic acid) at least 2.25 μgm. of coumarin (including coumaric acid to some extent) per seed were found to leach out during a 24-hour period. This estimate was derived by a subtraction of the somewhat crude "blank" quantity provided by parallel experiments on alfalfa leachates. This blank was probably too high for two reasons: (*a*) the analyses were made by the colorimetric method, and (*b*) it was observed that the green coloring matter of alfalfa leachates dissolved to an appreciable extent in ether; this is in contrast with the apparent insolubility of the pigments of sweetclover leachates.

It would be interesting to determine whether this leached coumarin has any relation to the germination of sweetclover seeds.

Discussion

A concentration of 5 p.p.m. of coumarin inhibited root growth of both alfalfa and Common Biennial White sweetclover seedlings by approximately 50% as compared with the controls. Alfalfa roots placed on agar containing 50 p.p.m. of coumarin did not elongate at all. Sweetclover roots placed on agar containing 50 p.p.m. of coumarin, however, showed only about 10% less growth than those on agar containing 5 p.p.m. of coumarin. This suggests that after a certain threshold concentration is reached, sweetclover roots may possess a "protective" mechanism which tends to offset the effect of an increase in concentration of the inhibitor.

In view of the sensitivity of the roots to 5 p.p.m. of coumarin, the term "protective" is applicable only after the threshold concentration has been reached.

From consideration of the importance attached to the reactivity of the double bond, melilotic acid, which is saturated but otherwise closely related to coumarin, would probably not be expected to have inhibitory activity. Data presented by BRINK (**5**) and those obtained in the present study, however, indicate inhibitory activity of melilotic acid and signify that such activity is seemingly not dependent upon unsaturation in the molecule. Further, BRINK found that the lactone of melilotic acid (coumarin with the 3,4 positions hydrogenated) also inhibited the growth of sweetclover and alfalfa roots.

In the experiments reported here the inactivity of o-coumaric acid at concentrations of 5 and 50 p.p.m., in contrast with the inhibitory effect of coumarin at these concentrations, may be explained if it is assumed that only the undissociated form of the acid is effective. Calculation involving the dissociation constant of o-coumaric acid, 2.14×10^{-5}, shows that only about 7% of the acid would be in the undissociated form in agar with a pH of 6.0. Thus, based on the assumption that only the undissociated form is effective, only about 3.5 p.p.m. of active substance would be present at a concentration of 50 p.p.m. of o-coumaric acid. This would tend to make the inhibitory effect of o-coumaric acid nearly similar to that of coumarin.

The activity of o-coumaric acid suggests that the lactone configuration also may be unessential in the structure of the inhibitor molecule. It would not seem likely that o-coumaric acid changed to coumarin in the medium during the course of the experiment. This possibility should be investigated, however.

No marked longitudinal gradient of coumarin concentration was found in roots of sweetclover in contrast to the sharp increase of scopoletin concentration with distance from the tip in roots of oat seedlings (**9**). In addition to this difference, the average concentration of free coumarin (on a fresh-weight basis) is at least five times that of scopoletin in oat roots. The discrepancy between the concentrations of the two substances is accentuated by the consideration, not discussed by GOODWIN and KAVANAGH (**9**), that the extraction method they used probably extracted the total scopoletin content, that is, scopoletin from the glycoside, scopolin (if present), in addition to the free substance.

As a result of the concentration gradient of scopoletin, this substance is present in the growing region of the oat root in a concentration which, if expressed per gram of fresh weight, is appreciably lower than any inhibitory concentration of externally applied scopoletin, expressed per gram of agar. Compared on the same basis, the concentration of coumarin in sweetclover roots is, in contrast, at least several times higher than that required for inhibition when applied externally. If it is assumed that free coumarin is equivalent to physiologically active coumarin, it might be suggested that the growth rate of sweetclover seedling roots may in part be a result of "normal" internal inhibition by coumarin. The fact that sweetclover roots usually have an appreciably slower growth rate than alfalfa roots tends to support this suggestion. The marked sensitivity, however, of sweetclover roots to a concentration of 5 p.p.m. of coumarin makes doubtful the assumption that free coumarin is equivalent to physiologically active coumarin.

In addition, differences between these two species other than the presence or absence of coumarin emphasize the speculative nature of these considerations.

Finally, in any discussion of the role of coumarin in the physiology of growth of sweetclover, it is necessary to consider the implication of the successful breeding program which has developed strains of sweetclover with low coumarin content (11). This would seem to indicate that a high degree of integration of coumarin in physiological processes of sweetclover is questionable. In this connection, it would be interesting to compare the growth rate of low-coumarin sweetclover roots with those of high-coumarin roots.

Summary

1. Melilotic acid and o-coumaric acid inhibited cell elongation in the *Avena* coleoptile curvature test. Coumarin had no activity under the conditions employed.

2. All three compounds inhibited root growth of sweetclover and alfalfa seedlings. It is suggested that perhaps neither unsaturation nor lactone configuration is essential for their inhibitory activity.

3. The total length increment of roots of sweetclover and alfalfa seedlings grown on agar containing 5 p.p.m. of coumarin was about 55 and 40%, respectively, of the control. At a concentration of 50 p.p.m. of coumarin the increment of sweetclover roots was further decreased by only about 10%, whereas alfalfa roots placed on such agar did not grow at all.

4. The concentration of free and bound coumarin present in sweetclover roots was quantitatively determined. The average concentration of free coumarin was about 74 μgm/gm of fresh weight. Coumaric acid may account for 20% of this quantity. No longitudinal concentration gradient of coumarin was found in the roots of sweetclover seedlings. The possible role of coumarin as a natural growth inhibitor is discussed.

5. During the imbibition and subsequent periods of leaching of sweetclover seeds, coumarin was found in the leachate in quantities proportionate to the length of leaching time. During a leaching period of 24 hours, about 2 μgm. of coumarin were leached out per seed.

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DEPARTMENT OF BOTANY
UNIVERSITY OF CHICAGO
CHICAGO 37, ILLINOIS

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RELATION OF IRRIGATION PRACTICE TO THE DIFFERENTIATION AND DEVELOPMENT OF APRICOT FLOWER BUDS

DILLON S. BROWN

Introduction

Nonirrigated apricot orchards frequently, though not always, bloom later and more erratically than those which have been irrigated. A water deficit during floral-bud differentiation and early development conceivably influences the ultimate opening of the buds in either one or both of two ways. First, it may delay differentiation and retard the rate of development of differentiated buds so that by blossom time they are not so advanced as buds on trees under more favorable soil-moisture conditions. Second, it may increase the intensity of the rest influence and prolong its effect so as to keep the buds dormant for a time even after temperatures become favorable for growth in the spring. It is the first of these possibilities, the effect of soil-moisture condition on the rate of flower-bud development, which is considered in this investigation.

Material and methods

During the summer of 1950 a study of apricot flower-bud development was begun in a differentially irrigated block of Royal apricots in the University Experimental Orchards near Winters, California. HENDRICKSON and VEIHMEYER (2) have described the block and presented a progress report on the effects of the treatments on tree growth and yield. In addition to the four treatments reported by them, a fifth unreplicated treatment was added in 1950 by utilizing one of the two plots of the original eighteen in the orchard which had not been used in the four-treatment-four-replicate layout. The irrigations applied during the summer of 1950 were as follows: plot A, May, July, August, and September; plot B, July and September; plot C, July; plot D, none; plot E, May.

The permanent wilting percentage was not reached in the top 6 feet of soil in plot A at any time during the season, although it was nearly reached in the top foot around the last of June. In plot B the wilting percentage was reached to a depth of 6 feet by the end of June and again by the first of September. In plot C the soil to this depth was at the wilting percentage by early June, and again by late August, remaining so for the rest of the season; the sixth foot was scarsely above the wilting percentage after early June. Plot D reached the wilting percentage to a depth of 6 feet in early June and remained so for the rest of the season. The soil moisture to this depth in plot E dropped to the wilting percentage by mid-July and continued there for the remainder of the season.

Preliminary observations of buds made under a dissecting microscope during July and August indicated differences in bud development between the dry and the wet plots. Beginning in early September bud samples were collected periodically and preserved for sectioning from the tier of plots (replicate) containing all five treatments. Samples were collected from all treatments except B; it was not sampled, since it appeared to be nearly like treatment A in tree and bud behavior. In sampling, one spur from among those which could be reached from the ground was collected at random from each of the eight trees in a plot. Three buds, located in a position where fruit buds are normally found, were preserved from each spur after the hard outer bud scales had been removed. Six to ten buds from each sample were sectioned and examined microscopically.

Samples were collected from plots A, C, D, and E on September 6, November 6, December 7, 1950, and January 2 and 15, and February 5 and 21, 1951. Full bloom occurred on March 12, 1951. Additional samples were collected on March 12 and 15. Those on March 12 were gathered primarily to determine the stage of development of buds which were dropping unopened as a result of the mild winter (1). Loose buds—those which were about to drop—were prevalent in plots A, C, and E. Loose buds were noticed on these plots at the time of the February 21 sampling, and a collection of such buds was also made then in addition to the regular sample.

There was a dearth of flower buds in treatment D, some trees in the plot being devoid of bloom. However, on a few trees there were some buds swelling which appeared to be late-developing flower buds; these made up the March 12 sample from plot D, whereas for the other plots the buds were those which were about to drop unopened. The samples on March 15 consisted of late-developing buds on treatments D and E. None was found on the other plots.

Results

Bud development was most rapid and uniform in plot A and successively slower and more erratic in plots C, E, and D. For comparative purposes various stages in the development of the buds have been assigned index numbers from 1 through 8. The appearance of the buds at these stages is shown in figures 1–14. A comparison of the development of the buds in the different treatments using these index numbers is presented in table 1.

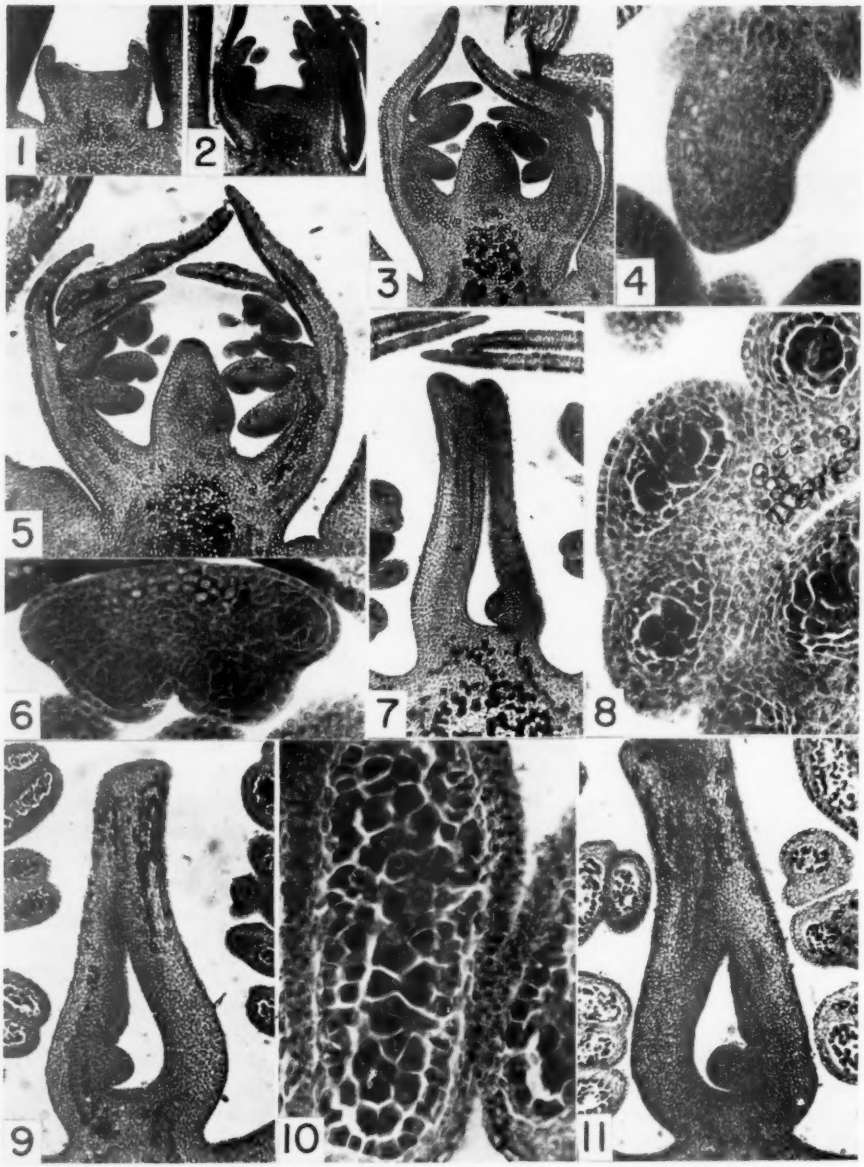
The buds on the trees of treatment C were less advanced than those of treatment A until early December, by which time most of them had attained about the same stage of development as A. The buds of C were somewhat more variable in development than those of A; even in January and early February both stages 5 and 6 could be found readily in plot C when most of the plot A buds were at stage 6 (figs. 7–10). During the course of sampling the trees it was noticed that there were fewer flower buds per node on the spurs from the C plot. At least two flower buds and frequently three per node were typical of plot A, whereas there were many nodes on the C spurs with only one flower bud (accompanied by two shoot buds) and only a relatively few with three.

The buds of treatment E were even slower in development than those of treatment C. It was mid-January to early February before nearly comparable stages could be found in plots A, C, and E. Development of the buds of plot E was much more variable than for plot C; at the December sampling, for example, buds ranging in development from stage 2 to stage 5 were found in plot E, while those of C were at stage 5, or nearly so (figs. 2–8). A high proportion of the nodes on the plot E spurs had only one flower bud, and only a few had three.

The buds of plot D were very much retarded. In fact, it was not until January 15 that a bud was found which was as advanced as those in treatment A were on October 5 (Figs. 5, 6). Nevertheless, there were some buds which were as fully advanced as those in the other plots of February 21 (figs. 13, 14), and some were in bloom with the other plots by March 12. Treatment D had very little bloom. Some trees were entirely devoid of bloom. The failure of the majority of the buds to differentiate undoubtedly accounts in part for the failure to detect more advanced stages earlier. With the low population, the chances of picking differentiated buds in random sampling were small, so that, although the buds which were preserved were taken from spur positions which normally produce flower buds, a high proportion of those collected from plot D were simply shoot buds.

There is no question, however, that there was a marked delay in the initiation of many flower buds on plot D. By late February, flower buds could be recognized by their larger size, though this had been possible on the other plots as early as September. On March 12, when the trees were in full bloom, and again on March 15 swollen buds were found on those trees of plot D which had a scattering of normal bloom. As shown in table 1 and figures 15–18 these buds varied in development from stages comparable to those found on plot A in October (figs. 3–6) to those comparable to plot A on February 21 (figs. 13, 14). On plot E also some late-differentiating buds were found on March 15. None sectioned, however, was less advanced than those of plot A on February 21.

It was not always possible to tell the late-differentiating buds by external appearances; many of them looked like



FIGS. 1–11.—Apricot flower buds: fig. 1, stage 1, sepal and early petal initials, plot E, Sept. 6, 1950; fig. 2, stage 2, sepal and petal primordia more advanced than in stage 1, plot A, Sept. 6, 1950; fig. 3, stage 3, all floral parts readily distinguishable, plot A, Oct. 5, 1950; fig. 4, stage 3, anther without evident sporogenous tissue, plot A, Oct. 5, 1950; fig. 5, stage 4, all floral parts more advanced than stage 3, plot A, Oct. 5, 1950; fig. 6, stage 4, anther with sporogenous initials evident, plot A, Oct. 5, 1950; fig. 7, stage 5, pistil and young ovule as in plot A, Nov. 6–Dec. 7, 1950; fig. 8, stage 5, anther with pollen mother cells, plot A, Nov. 6–Dec. 7, 1950; fig. 9, stage 6, pistil and ovule as in plot A, Jan. 2–15, 1951; fig. 10, stage 6, portion of anther with pollen mother cells as in plot A, Jan. 2–15, 1951; fig. 11, stage 7, pistil and ovule as in plot A, Feb. 5, 1951. Figs. 1, 2, 3, 5, 7, 9, 11, × 57; figs. 4, 6, 8, 10, ×247.

swelling shoot buds. When examined under a dissecting microscope, many of the inner scales of the late-differentiating buds were more leaflike than usual. The buds appeared as though they had started growth as normal shoot buds but had differentiated after some expansion of the axis had begun. As shown by the anther to the left in figure 18, many of the anthers were petaloid. Flowers which opened from these buds had an abnormally large number of petals (were "double" flowers) and had long pedicels which frequently bore one or more small leaves.

The collections made on February 21 and March 12 of buds which were about to drop unopened showed a marked deterioration of either the anthers, or pistil, or both (figs. 19–21). The anthers were frequently completely collapsed and shriveled. If not collapsed, the pollen grains were thin walled and did not stain deeply; many were empty (fig. 21). Such flower-bud deterioration, resulting from the mild winter, occurred on all plots and was in no way influenced by the irrigation treatments.

Discussion

TUFTS and MORROW (**3**) quoted the previously unpublished conclusion of WIGGANS (**4**) that irrigation has a tendency to retard fruit-bud differentiation and development. The results herein reported are in direct opposition to such a conclusion. Since WIGGANS compared samples from irrigated and nonirrigated orchards in different parts of the state, other variables may have influenced bud development and been misleading as to the effects of irrigation.

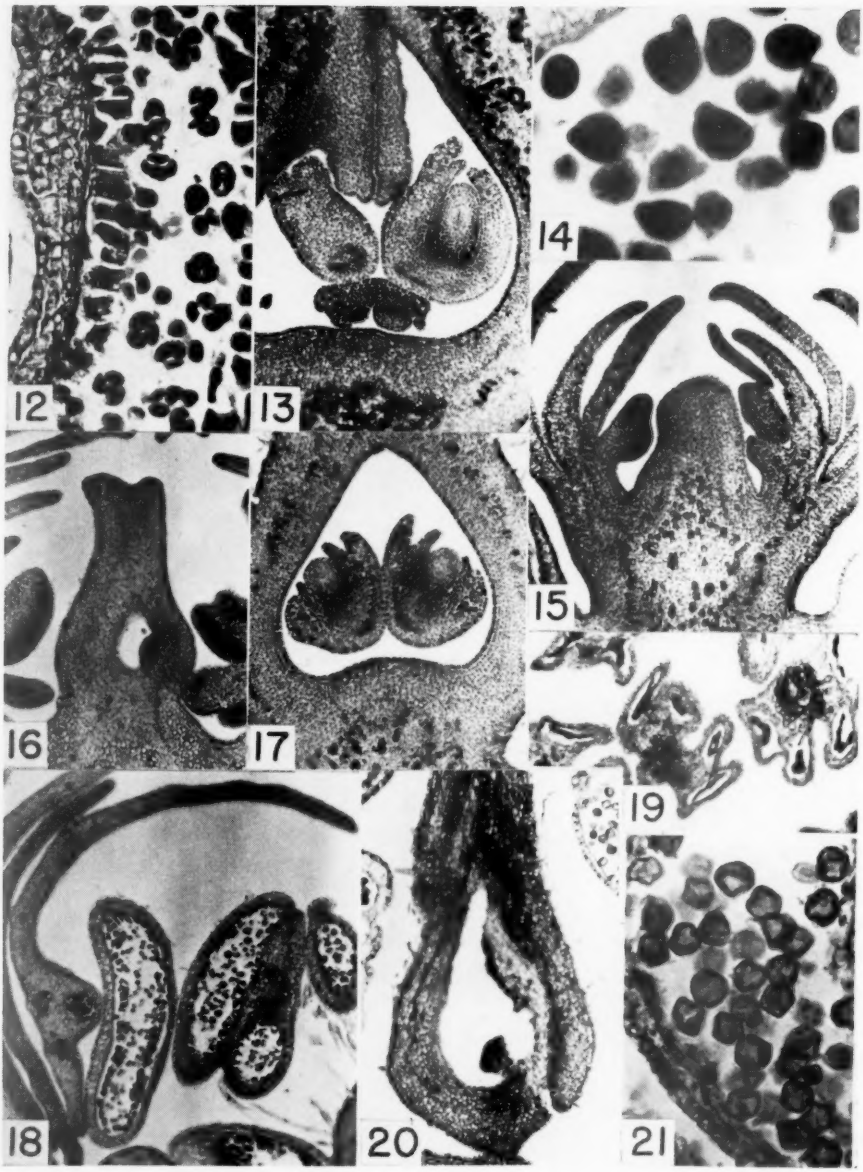
Since the winter of 1950–51 was one of the mildest on record, especially until late December, it is possible that any retarding effects of a water deficit on bud development may have been accentuated. However, since all trees in the com-

TABLE 1

STAGE OF DEVELOPMENT* ON DIFFERENT DATES OF APRICOT FLOWER BUDS FROM IRRIGATION PLOTS

Date	Treatment			
	A	C	E	D
9/6/50	2	−2, 2	1	−1, 1
10/5/50	3, 4	3, 3+	1	−1
11/6/50	−5, 5	3, 4+	1, 3	−1
12/7/50	5, 5+	−5, 5	2, 3, −5, 5	−1
1/2/51	−6, 6	5, 6	5	1
1/15/51	6	5, 6	5, 5+	1, 2, 4
2/5/51	6+, 7	6, 6+	5+, 6	−1
2/21/51	8	8	8	1, 6, 8
3/12/51	Bloom	Bloom	Bloom	2+, 6, 7+, Bloom
3/15/51			8	3, 7, 8
	Number of irrigations, 1950			
Before harvest	1	0	1	0
After harvest	3	1	0	0

* Stages of development, graded from 1 to 8, illustrated in figs. 1–14.



FIGS. 12–21.—Apricot flower buds: fig. 12, stage 7, portion of anther showing tetrads, plot A, Feb. 5, 1951; fig. 13, stage 8, well-developed ovule as in plot A, Feb. 21, 1951; fig. 14, stage 8, well-developed pollen grains, plot A, Feb. 21, 1951; figs. 15–17, late-developing flower buds, March 12–15, 1951, compare with figs. 3, 7, 13; fig. 18, anthers in late-developing flower bud, March 12–15, 1951, showing petaloid anther to left with pollen mother cells in locule, next anther with pollen grains, next with tetrads; fig. 19, anther deterioration in bud about to drop following mild winter, Feb. 21, 1951; fig. 20, pistil and ovule deterioration in bud about to drop, Feb. 21, 1951; fig. 21, thin-walled, deteriorating pollen grains in bud about to drop, Feb. 21, 1951, compare with fig. 14. Figs. 13, 15–20, ×57; figs. 12, 14, 21, ×247.

parison were subject to the same climatic conditions, there is no question but that the irrigation practice was a factor in determining the time of differentiation. It also must have influenced the amount of differentiation and the rate of development of differentiated buds. Since plot C, which was irrigated once after harvest in 1950 though not before, was better than plot E, which was irrigated before harvest but not after, the importance of adequate soil moisture during late summer is demonstrated. It is also sharply indicated by the fact that plot B (not sampled), which received two irrigations after harvest though none before, is producing better than plot C and is nearly as good as plot A (table 2).

TABLE 2

AVERAGE YIELD IN POUNDS PER TREE FOR TIER OF REPLICATE FROM WHICH BUD SAMPLES WERE TAKEN

Treatment	Year		
	1949	1950	1951
A	270.1	261.4	168.6
B	236.6	209.0	177.5
C	167.1	199.0	147.4
D	199.6	43.8	10.8
E	124.8	232.0	62.9

The soil moisture should not be at the permanent wilting percentage for any prolonged period during July, August, and September if satisfactory, timely, flower-bud differentiation in the apricot is to occur. The slowest and least differentiation occurred in plot D, which remained at the wilting percentage after early June. Plot E, which was only a little better than plot D in the uniformity and timeliness of bud development, remained at the wilting percentage after mid-July. Plot C, which was considerably better in bud behavior than either D or E, was at the wilting percentage for a short period during harvest (June) prior to its one irrigation (July) and continuously at the wilting percentage only after late August. Plot B, the buds of which were not sampled, was at the wilting percentage for short periods in June and late August but apparently was not adversely affected, since the time and amount of bloom were comparable to plot A, which always was amply supplied with moisture.

The importance of timely irrigation applications on bud differentiation and development is borne out by the yield records of the trees. Table 2 gives the average yield per tree for the 1949, 1950, and 1951 seasons for the tier of replicates from which the bud samples were obtained. A generally lower yield on all plots in 1951 can be accounted for in part by the bud drop resulting from the mild winter. However, a fairly heavy crop would normally have been expected on plot D following the light crop of 1950. Actually, six of the eight trees in the D plot sampled did not produce any crop; flower buds were not differentiated even following the light crop of 1950. Some decrease in yield in treatment E might have been expected following a heavy crop in 1950, but the decrease that actually occurred is much more than normally would have been anticipated and cannot be accounted for by shedding of flower buds following a mild winter. The conclusion is inescapable that for plots E and D, at least, a water deficit in late summer markedly reduced flower-bud differentiation as well as delayed the differentiation and development of the buds.

Summary

Prolonged periods of dry-soil conditions during July, August, and September because of no or infrequent irriga-

tions affected Royal apricot trees by (*a*) limiting the number of flower buds differentiated; (*b*) delaying the time of differentiation of many of the flower buds; and (*c*) slowing the rate of development of buds which were differentiated at about the normal time.

The assistance of Mrs. THELDA BARTLEM in the preparation of the microscopic slides is gratefully acknowledged.

DIVISION OF POMOLOGY
UNIVERSITY OF CALIFORNIA
DAVIS, CALIFORNIA

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OVARY-WALL DEVELOPMENT AS INFLUENCED BY GROWTH-REGULATORS INDUCING PARTHENOCARPY IN THE CALIMYRNA FIG

JULIAN C. CRANE

Introduction

It is desirable to induce parthenocarpic development of Calimyrna figs and thus eliminate the need for caprification (cross-pollination) by the fig wasp, *Blastophaga psenes* L. (**3**). During the last 5 years several plant growth-regulating compounds have been demonstrated to induce parthenocarpy in this variety (**2, 3, 4**). Histological investigations have revealed pronounced modifications from the normal development and composition of the ovary wall of achenes within the syconium, depending upon the growth-regulator used (**1**). Figs produced with para-chlorophenoxyacetic acid have become known commercially to the trade as "miracle seedless figs," since they do not contain the completely developed achenes that characterize pollinated figs. Although the industry has adopted on a small scale the use of para-chlorophenoxyacetic acid as a spray to induce parthenocarpy, the figs produced by this method apparently have a limited market. Processors have been slow to accept seedless figs because of possible consumer resistance to the smooth-textured products made with them. Experimental investigations, therefore, have been focused on the testing of various growth-regulating substances in an attempt to find one which induces the production of parthenocarpic figs containing achenes with a sclerified endocarp and which does not injure the foliage. Of several compounds tested during the 1951 season, one was found to meet these requirements.

Material and methods

The methods followed were essentially the same as those previously reported (**2, 3**). The investigation was conducted in a commercial orchard under standard practices of production. To exclude the

pollinating fig wasp, it was necessary, therefore, to cover the test branches with muslin bags during the period of caprification. All test branches were bagged on June 11, 1951, and, with the exception of a few minutes during treatment, remained covered until July 3. Commercial caprification was begun on June 12, and the last application of caprifigs in the orchard was made on June 30.

Crystalline benzothiazol-2-oxyacetic acid was treated with a sufficient quantity of concentrated ammonium hydroxide to make a thin paste and then was dissolved in water. Hydrochloric acid was added to the stock solution to adjust it to pH 5.5. Various dilutions of this stock solution were applied on June 18 with a continuous-delivery type, hand-operated, insect-spray gun to individual branches that were selected for uniformity of vigor and number of syconia. Five branches were used per treatment, each bearing at least five syconia at the time of application. Both the leaves and the young syconia were wet with the spray material to the point of runoff. Fifty unbagged branches that were selected at random throughout the orchard served as controls for the determination of the percentage of fruit-set as a result of caprification.

Syconia of the same age were selected for histological study: only figs borne at the most proximal nodes on wood of the current season were used. The samples consisted of five syconia of each treatment. A segment (1 cm. square) through one side of each syconium was removed from a standard position toward the distal end. The material was killed and fixed in formalin–acetic-alcohol, prepared according to the usual paraffin method, sectioned perpendicularly to the epidermis at 10 μ, and stained in safranin and fast green.

TABLE 1

EFFECTS OF BENZOTHIAZOL-2-OXYACETIC ACID APPLICATION ON CALIMYRNA FIG

Treatment	% set of syconia	% syconia maturing early	Injury
Benzothiazol-2-oxyacetic acid* (concentration, p.p.m.):			
100	100	20	No leaf injury
500	100	47	Terminal leaves killed
1000	100	53	Leaves killed and minor wood injury
1500	100	77	Leaves killed and moderate wood injury
2000	100	100	Leaves killed and severe wood injury
Caprified controls	87		

* Supplied by American Cyanamid Co.

Results and discussion

In contrast to an 87% set of syconia that were caprified, benzothiazol-2-oxyacetic acid induced a 100% set of syconia at each of the concentrations listed in table 1. In addition to the high degree of effectiveness of this compound for inducing parthenocarpy, it also promoted an acceleration in growth rate to the extent that some of the syconia matured approximately 3 weeks after the date of spraying or about 6 weeks before the time of maturity of caprified syconia. There was a positive correlation between concentration from 100 to 2000 p.p.m. and the percentage of syconia that matured early. No observable symptoms of

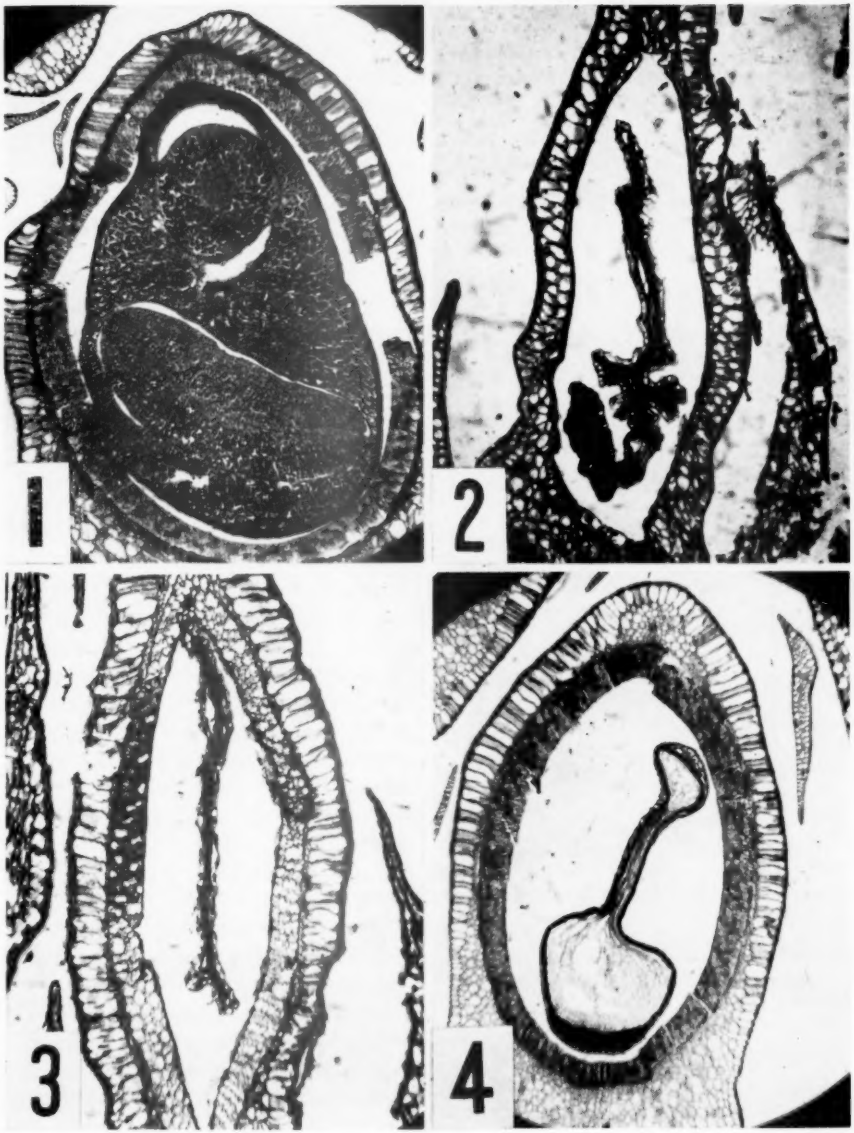
injury were apparent on the branches sprayed with the 100-p.p.m. solution, but at higher concentrations injury occurred in the form of chlorosis and ultimate death of the leaves and necrotic areas on the current season's wood.

From their external appearance it was difficult, if not impossible, to distinguish between caprified figs and those induced parthenocarpically with benzothiazol-2-oxyacetic acid. The rather pronounced ribbing which characterizes figs induced with para-chlorophenoxyacetic acid (**4**) was not present in those induced with benzothiazol-2-oxyacetic acid. Figs induced with the latter compound appeared identical with caprified figs as far as achene development was concerned, but the color of the pulp in the two types was markedly different. The pulp in mature caprified figs was a typical strawberry-red color, while that in the parthenocarpic syconia was a light amber-yellow, the same color that develops in parthenocarpic figs induced with para-chlorophenoxyacetic acid (**4**). The formation of red pigmentation is apparently associated with growth of the embryo, since the lack of embryo development in achenes from syconia induced with benzothiazol-2-oxyacetic acid was the only characteristic distinguishing them from achenes in caprified syconia. It had formerly been thought that formation of the red pigment was associated with ovary-wall development (**4**).

Histological examinations of achenes in syconia induced with benzothiazol-2-oxyacetic acid revealed the endocarp tissue to be completely sclerified (fig. 4). In fact, the three component layers—exocarp, mesocarp, and endocarp—of the ovary wall of achenes from syconia induced with benzothiazol-2-oxyacetic acid appear identical with those of achenes in caprified syconia (compare figs. 1 and 4). For purposes of comparison previously published photomicrographs (**1**) of achenes from parthenocarpic syconia induced with gamma-(indole-3)-n-butyric acid (fig. 2) and para-chlorophenoxyacetic acid (fig. 3) are presented.

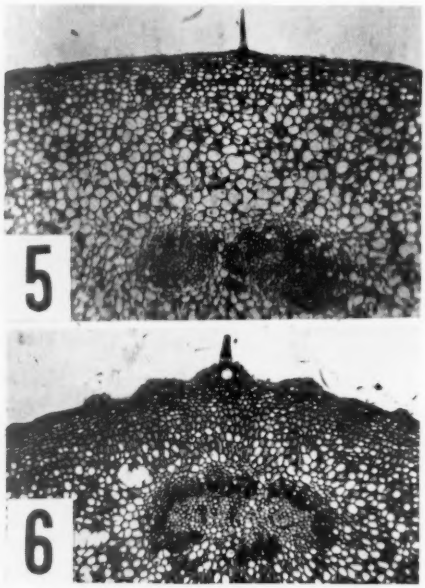
Depending upon the growth-regulator used to induce parthenocarpy, distinctly different achenes can be produced. The following three deviations from normally pollinated syconia are possible in the Calimyrna fig: (*a*) syconia containing achenes in which the endocarp is lacking (induced with gamma-[indole-3]-n-butyric acid); (*b*) syconia containing achenes in which the endocarp is present but not sclerified (induced with para-chlorophenoxyacetic acid); and (*c*) syconia containing achenes in which the endocarp is present and completely sclerified (induced with benzothiazol-2-oxyacetic acid). Achenes in none of these types contain an embryo. Thus the setting and the subsequent development of Calimyrna figs as a result of application of benzothiazol-2-oxyacetic acid suggest that the chemical properties of this synthetic plant growth-regulator more nearly approach those of the naturally occurring hormone in this variety than do the properties of other growth-regulating compounds which have been found to induce parthenocarpy.

Although identical development of the various tissues composing the ovary wall of achenes occurred in caprified syconia and in those induced with benzothiazol-2-oxyacetic acid, pronounced differences exist in the outer epidermal layer of the two types of syconia. The external surface of syconia induced parthenocarpically with benzothiazol-2-oxyacetic acid is smooth (fig. 5), while the surface of caprified syconia is undulated (fig. 6). The undulations are particularly pronounced in the areas around and directly



FIGS. 1–4.—Typical achenes from caprified and from parthenocarpic Calimyrna syconia induced by growth-regulators. Fig. 1, from caprified syconium showing exocarp, mesocarp, and sclerified endocarp of ovary wall with partially developed embryo. ×50. Fig. 2, from syconium induced with gamma-(indole-3)-n-butyric acid. Note absence of endocarp tissue and collapsed nucellus. ×67. Fig. 3, from syconium induced with para-chlorophenoxyacetic acid. Endocarp tissue is present but not sclerified. This was only achene examined which had a small portion of endocarp sclerified, represented by dark area at left. ×67. Fig. 4, from syconium induced with benzothiazol-2-oxyacetic acid. Note endocarp is as fully sclerified as in Figure 1. ×50.

adjacent to the epidermal trichomes. The basal or foot portion of the trichomes on caprified syconia are more deeply imbedded in the epidermal layer than in the case of parthenocarpic syconia. The epidermal cells of parthenocarpic syconia varied in radial section from square to rectangular, with the long axis of the rectangular cells parallel to the surface of the syconium. These cells in caprified syconia, on the other hand, were rectangular in radial section, with the long axis perpendicular to the surface.



FIGS. 5, 6.—Radial sections through epidermis and cortex of Calimyrna fig syconia showing unicellular trichomes. ×61. Fig. 5, parthenocarpic syconium induced with benzothiazol-2-oxyacetic acid. Fig. 6, caprified syconium showing undulated epidermis.

Summary

1. Aqueous sprays of the ammonium salt of benzothiazol-2-oxyacetic acid, applied at concentrations ranging from 100 to 2000 p.p.m., induced 100% of unpollinated Calimyrna fig syconia to develop parthenocarpically. This compound also promoted an acceleration in growth rate, so that, depending upon concentration, a certain percentage of the treated syconia matured 6 weeks before the maturity date of caprified syconia. No observable symptoms of injury accompanied applications of 100 p.p.m., but above this concentration injury occurred in the form of chlorosis and ultimate death of the leaves and necrotic areas on the current season's wood.

2. With the exception of differences in color of the pulp and lack of embryos in achenes from parthenocarpic syconia, the latter could not be distinguished from syconia that had been caprified.

3. In contrast to a complete absence of endocarp tissue in achenes from parthenocarpic syconia induced with gamma-(indole-3)-n-butyric acid and the presence of unsclerified endocarp tissue in achenes from syconia induced with para-chlorophenoxyacetic acid, histological investigations revealed this tissue to be completely sclerified in achenes from syconia induced with benzothiazol-2-oxyacetic acid.

4. In addition to differences in shape and arrangement of cells composing the epidermal tissue of the two types of syconia, the external surface of syconia induced parthenocarpically with benzothiazol-2-oxyacetic acid was smooth, while that of caprified syconia was undulated.

Appreciation is expressed to Mrs. THELDA BARTLEM, who did the microtechnique work in this study.

DIVISION OF POMOLOGY
UNIVERSITY OF CALIFORNIA
DAVIS, CALIFORNIA

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RESPONSE OF BLACK CORINTH GRAPES TO APPLICATIONS OF 4-CHLOROPHENOXYACETIC ACID

ROBERT J. WEAVER

Introduction

Girdling has been the accepted practice for obtaining compact clusters with large seedless berries in the production of Black Corinth grapes. Recently it has been demonstrated in California (**3, 4**) and Australia (**1**) that applications of 4-chlorophenoxyacetic acid to flowering clusters on Black Corinth vines induce effects similar to girdling. Often, however, use of the compound results in development of hard seeds (**3, 4**), which would make these grapes unacceptable for production of currants. One objective of the experiments, performed in 1951 and reported here, was to determine whether a growth-regulator could induce development of compact clusters of berries containing no hard seeds. Other experiments concerning the relation of applications of growth-regulator and girdling to development of Black Corinth grapes are also reported.

Material and methods

Use was made of an irrigated vineyard located 1 mile west of Davis, California. In most cases, Black Corinth vines were pruned to four canes, each usually bearing from eight to twelve buds. Each cane was tied horizontally on one wire of a three-wire, flat-top trellis. On May 23 all clusters except those on the canes were removed. There were about fifteen clusters per cane, although there was some variation.

Sufficient ammonia was added to dissolve an aqueous suspension of 4-chlorophenoxyacetic acid. Dreft was used as a wetting agent. Clusters of flowers or fruits were immersed momentarily into one of the solutions. The unit of treatment in dipping experiments was usually all clusters on one cane. Replicate canes were selected at random from the vines used.

For spray applications of growth-regulator, foliage and clusters of individual canes were thoroughly wet with a Craftsman sprayer. Cardboards were used to confine spray to the intended cane.

A strip of bark $\frac{1}{8}$ inch in width was removed near the base of girdled canes with girdling pliers.

At harvest berries removed from similar locations of clusters were thoroughly mixed, and the weight of two hundred was obtained as an indication of relative size of the berries. The number of berries containing hard seeds was then ascer-

tained. Hard seeds were gritty and could not be easily crushed by pressure of a fingertip. The remaining berries were crushed, and the percentage of total soluble solids in the juice was determined with a Balling hydrometer. A hand refractometer was used in a few instances when there was only a small amount of juice. Total acidity was determined by adding 50–100 ml. of distilled water to 10 ml. of juice and then titrating with 0.133*N* NaOH, using phenolphthalein as an indicator. The results are expressed as grams of acid calculated as tartaric acid per 100 ml. of juice, which is approximately the percentage of acid.

Experimentation and results

RESPONSE OF VINES TO SPRAY APPLICATIONS OF GROWTH-REGULATOR AT VARYING CONCENTRATIONS.—The purpose was to determine the range of concentrations of growth-regulator resulting in largest berries and clusters. Vines were sprayed with 4-chlorophenoxyacetic acid at 10, 25, 50, 100, or 300 p.p.m. on May 28, after calyptras had fallen and stamens dried. Four canes were used for each chemical treatment. Four canes were girdled, and four were untreated controls.

By June 8 many berries on untreated, control clusters had set, although most flowers in the shaded interior part of the vine had shattered. The growth-regulator at 10 or 25 p.p.m. resulted in a high percentage of set of berries and in no injury to foliage. The compound at 50 p.p.m. resulted in good set of berries but caused some bending of shoot tips. These tips were straightening, although young leaves were still crumpled. Berries treated with the compound at 100 p.p.m. had not enlarged, and leaves on the apical 4–5 inches of these shoots were crumpled. The rachises of clusters sprayed with the compound at 300 p.p.m. were twisted and brittle, and berries were yellow and had not enlarged. Tendrils and the apical 4 inches of most of these shoots were dead. Splits and calluses were found on many parts of some of these shoots. The young leaves were crumpled and dying, even some of the largest leaves were crumpled.

By June 14 foliage sprayed with the compound at 300 p.p.m. had turned yellow and had become very crinkled. Cracks on these shoots were $\frac{1}{2}$–2 inches or more long; some extended the length of the internode and were $\frac{1}{4}$ inch wide. By June 28 foliage and fruit on these canes were dead.

TABLE 1

DATA AT HARVEST (AUGUST 20) FOR BLACK CORINTH GRAPES FROM VINES SPRAYED ON MAY 28 WITH 4-CHLOROPHENOXYACETIC ACID. AVERAGE OF FOUR REPLICATE CANES

Treatment, concentration of acid (p.p.m.)	Weight of 200 berries (gm.)	% berries containing hard seeds	Degree Balling reading	% acid
50 (not girdled)	88.2	17.3	22.6	0.76
25 (not girdled)	78.4	11.5	21.5	.85
10 (not girdled)	70.5	5.5	23.2	.96
0 (not girdled)	52.4	2.7	25.0	.90
0 (girdled)	113.2	2.8	21.3	0.85

Fruit was harvested on August 20 (table 1). The compound at 10 or 25 p.p.m. was apparently not injurious to foliage. Some canes sprayed with growth-regulator at 50 p.p.m. had thickened and developed calluses, although foliage and fruit developed normally. Foliage and clusters sprayed with the compound at 100 or 300 p.p.m. were usually dead, and there were large cracks on dead shoots.

Large berries and compact clusters developed when the growth-regulator was used in the range from 10 to 50 p.p.m. (table 1, fig. 1). Berries were smaller, however, than those on unsprayed canes

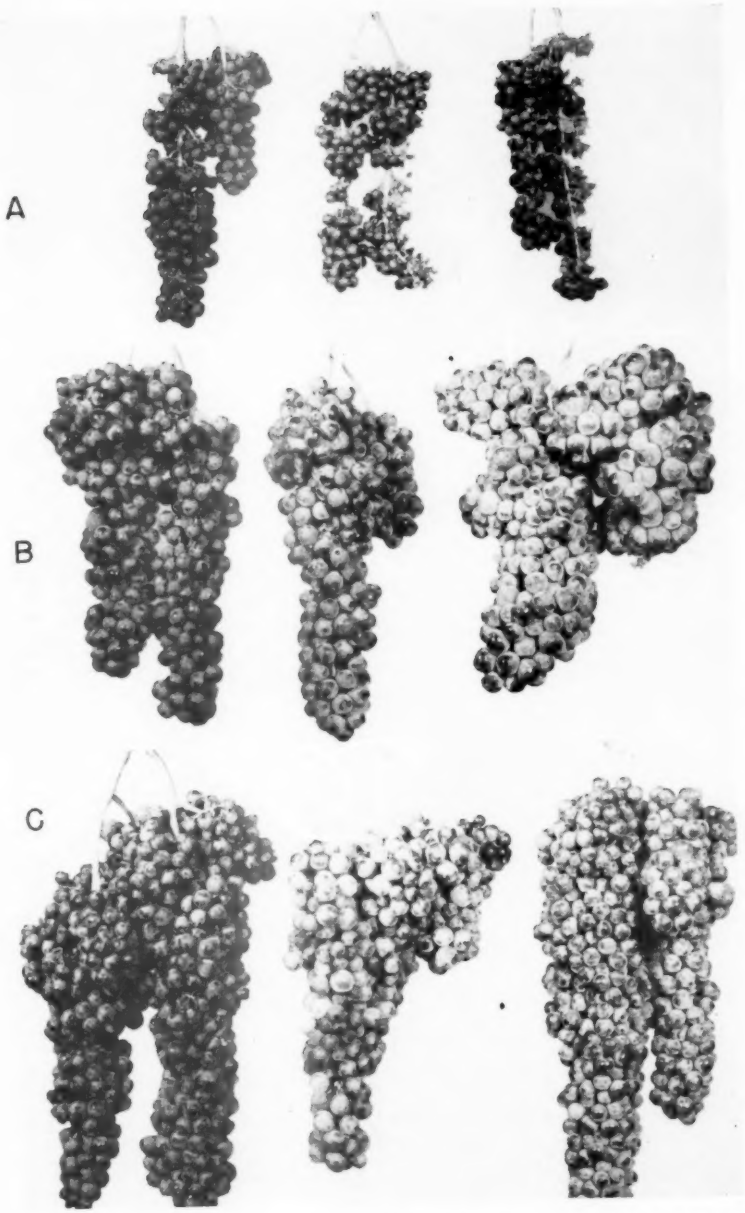
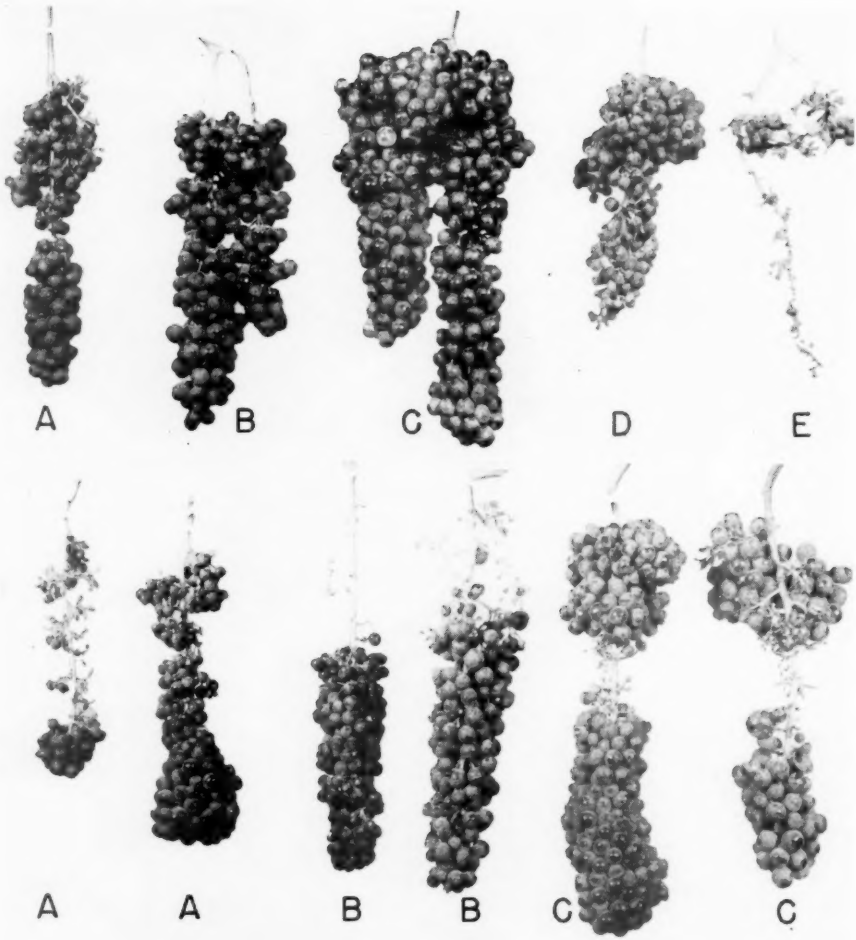


FIG. 1.—Black Corinth grapes 84 days after treatment. *A*, not sprayed, not girdled; *B*, not sprayed, girdled; and *C*, sprayed with 4-chlorophenoxyacetic acid at 25 p.p.m., not girdled. Note that effects of girdling and growth-regulator each resulted in compact clusters. (Photographed August 20, 1951.)



FIGS. 2, 3.—Clusters of Black Corinth grapes treated with 4-chlorophenoxyacetic acid. Fig. 2 (*above*), 83 days after dipping in acid at concentrations of 10 (*B*), 25 (*C*), 300 (*D*), or 1000 p.p.m. (*E*). Note that largest cluster and berries were produced at 25 p.p.m., that treatment at 300 p.p.m. resulted in a loose cluster, especially at apex, and that highest concentration, 1000 p.p.m., was lethal (*E*). *A*, control. (Photographed August 20, 1951.) Fig. 3 (*below*), 86 days after apical end (*A*), apical half (*B*), or apical and basal parts (*C*) were treated with acid at 50 p.p.m. Note that berries have usually enlarged only where acid was applied. (Photographed August 23, 1951.)

that were girdled. Probably because of their greater weight, berries from girdled vines had the lowest degree Balling reading.

RESPONSE OF CLUSTERS TO DIP APPLICATIONS OF GROWTH-REGULATOR AT VARYING CONCENTRATIONS.—Clusters were dipped into solutions of 4-chlorophenoxyacetic acid at 10, 25, 50, 100, 300, or 1000 p.p.m. on May 29, one cane per treatment. One cane was girdled, and another was the untreated control.

TABLE 2

DATA AT HARVEST (AUGUST 20) FOR BLACK CORINTH GRAPE CLUSTERS DIPPED IN 4-CHLOROPHENOXYACETIC ACID ON MAY 29

Treatment, concentration of acid (p.p.m.)	Weight of 200 berries (gm.)	% berries containing hard seeds
300 (not girdled)	56.6	22.5
100 (not girdled)	107.6	14.0
50 (not girdled)	99.0	9.5
25 (not girdled)	99.9	9.5
10 (not girdled)	68.0	0.0
0 (not girdled)	54.9	0.0
0 (girdled)	105.5	0.0

Fruit was harvested on August 20 (table 2, fig. 2). Many berries had set on some untreated control clusters, although there was much variation. Some clusters dipped in growth-regulator at 10 p.p.m. were straggly, as many berries failed to enlarge (fig. 2*B*), probably because concentration of the acid was too low. Clusters treated with the compound at 25, 50, or 100 p.p.m. were compact, with their large berries resembling those from girdled vines. Clusters treated with acid at 300 p.p.m. were loose, especially in the apical parts, because many berries did not enlarge. Most clusters immersed in the highest concentration, 1000 p.p.m., of the compound, were dead.

The results obtained with dipping are very similar to those from spray treatments (table 1). Berries of dipped clusters were, as a rule, slightly larger. The percentage of hard seeds was somewhat proportional to the concentration of growth-regulator (**4**).

TIME OF SPRAYING OF GROWTH-REGULATOR IN RELATION TO DEVELOPMENT OF FRUIT.—Black Corinth vines were sprayed at various developmental stages in an attempt to obtain berries without hard seeds. Four canes were sprayed with 4-chlorophenoxyacetic acid at 25 p.p.m.

TABLE 3

DATA AT HARVEST (AUGUST 23) FOR BLACK CORINTH GRAPES FROM VINES SPRAYED AT VARYING TIMES WITH 4-CHLOROPHENOXYACETIC ACID AT 25 P.P.M. AVERAGES OF FOUR REPLICATE CANES

Time of treatment	Weight of 200 berries (gm.)	% berries containing hard seeds	Degree Balling reading	% acid
Sprayed May 24	96.7	71.8	20.9	0.69
Sprayed May 28	70.7	20.9	22.0	.74
Sprayed June 3	72.9	0.1	22.4	.77
Sprayed June 7	73.2	0.8	25.1	.74
Sprayed June 14	64.3	0.4	24.9	.71
Not sprayed, not girdled	35.8	0.3	24.2	.78
Not sprayed, girdled	74.2	0.0	19.3	0.86

on May 24, when about 70% of the total number of calyptras had fallen. Four unsprayed canes were girdled. Other applications were made in a similar manner on May 28, when most calyptras had fallen and stamens had dried; on June 3, when berries were about $\frac{1}{16}$ inch in diameter; on June 7; and on June 14, when berries were about $\frac{3}{16}$ inch in diameter.

Fruit was harvested on August 23 (table 3). June 3 was apparently about the earliest possible date in 1951 for spraying in order to obtain compact clusters with almost no hard seeds. Application of growth-regulator on June 7 also

resulted in large berries with practically no hard seeds. The degree Balling reading was usually higher with later spraying, but time of treatment had no consistent effect on percentage of acid.

EFFECT OF TIME OF DIPPING WITH GROWTH-REGULATOR AND TIME OF GIRDLING ON DEVELOPMENT OF BLACK CORINTH GRAPES.—Clusters on two replicate canes were dipped in 4-chlorophenoxyacetic acid at 50 p.p.m. on May 27.

TABLE 4

DATA AT HARVEST (AUGUST 14) FOR BLACK CORINTH GRAPES FROM CLUSTERS DIPPED IN 4-CHLOROPHENOXYACETIC ACID AT 50 P.P.M. OR FROM CANES GIRDLED AT VARIOUS TIMES. AVERAGES OF TWO REPLICATE CANES

Time of treatment	Weight of 200 berries (gm.)		% berries containing hard seeds	
	Dipped	Girdled	Dipped	Girdled
May 27	85.2	62.3	46.0	2.5
May 31	79.0	113.9	3.0	0.5
June 4	76.2	99.8	2.8	2.0
June 8	66.0	77.1	0.0	0.3
June 14	52.4	70.8	0.0	0.0
June 28	46.6	40.2	0.3	0.5
Untreated control	33.9	.....	0.7	.....

Two other canes were girdled. Similar treatments were done on later dates (table 4).

Fruit was harvested on August 14 (table 4), when about three-fourths of the total surface of the control fruit was blue. Peduncles and rachises of clusters of the last three dippings were split and callused. Many berries dipped on June 14 or 28 were small and green.

Dipping on May 31 or June 4 resulted in compact clusters with few berries containing hard seeds. Berries of dipped fruit were usually smaller, however, than those on corresponding girdled canes.

EFFECT OF GIRDLING AND TREATMENT WITH GROWTH-REGULATOR, SEPARATELY AND COMBINED, ON BLACK CORINTH FRUIT.—The purpose was to determine whether larger berries could be obtained by girdling and dipping in growth-regulator than by either means used separately. Clusters on four ungirdled canes were dipped in 4-chlorophenoxyacetic acid at 50 p.p.m. on May 28. Four canes were girdled and clusters dipped, four were girdled but clusters not dipped, and four canes were untreated controls.

Fruit was harvested on August 13 (table 5). Many berries had shattered from control clusters, but those remaining were quite large. Applications of growth-regulator resulted in thickened pedicels and in berries as large as those produced after both girdling and application of growth-regulator.

TABLE 5

SEPARATE AND COMBINED EFFECTS ON FRUIT AT HARVEST (AUGUST 13) OF GIRDLING AND DIP APPLICATIONS OF 4-CHLOROPHENOXYACETIC ACID AT 50 P.P.M. ON MAY 28

Treatment	Weight of 200 berries (gm.)	% berries containing hard seeds
Untreated control	64.4	.....
Girdled only	76.7	0.6
Growth-regulator only	104.2	28.9
Girdled and growth-regulator	96.6	15.0

APPLICATION OF GROWTH-REGULATOR TO PORTIONS OF BLACK CORINTH CLUSTERS.—Portions of clusters were treated on May 29 by dipping in or spraying with 4-chlorophenoxyacetic acid at 50 p.p.m. Spray was applied with a 1-quart, continuous-delivery type, fly-spray gun. Before spraying, strips of paper were wrapped around parts of clusters intended to be unsprayed. There were five treatments, six replicate clusters each: (*a*) apical end of clusters immersed, (*b*)

apical half of cluster immersed, (*c*) basal half of cluster sprayed, (*d*) middle part of cluster sprayed, and (*e*) apical and basal parts sprayed.

At harvest time, August 23, it was evident that only the parts treated with growth-regulator responded (fig. 3). Appearance of clusters indicated that little or none of the growth-regulator, or stimulus induced by it, moved from the treated areas. Response to the compound was indicated by an increased percentage of berry set; by larger berries, many with hard seeds; and by thickened pedicels.

Discussion

In these experiments when applications of growth-regulator were delayed for 7–10 days after full bloom, compact clusters containing very few berries with hard seeds developed. In 1951 the shatter of berries normally following flowering was relatively small and delayed. In years when extensive and rapid shatter of berries occurs, the period in which growth-regulator can be applied to result in large, seedless berries would be much shortened.

The fact that 4-chlorophenoxyacetic acid applied to clusters at flowering resulted in more berries with hard seeds than did later application agrees with results of a previous experiment (4). Delayed girdling resulted in a lower number of berries per cluster (2). JACOB (2) showed that Black Corinth grapes girdled at full bloom developed many more berries per cluster than did those girdled after berries were approximately one-fourth grown.

Summary

1. Clusters and foliage of Black Corinth grapes were sprayed with 4-chlorophenoxyacetic acid at concentrations of 10, 25, 50, 100, or 300 p.p.m. Large berries and compact clusters developed when the acid was applied in the range from 10 to 50 p.p.m. Dipping of clusters gave similar results.

2. Parts of Black Corinth vines were sprayed with 4-chlorophenoxyacetic acid at 25 p.p.m. on May 24, May 28, June 3, June 7, or on June 14. June 3 was apparently the earliest time to spray in 1951 to obtain compact clusters with almost no hard seeds. Dipping of clusters gave approximately the same results.

3. Dip applications of 4-chlorophenoxyacetic acid at 50 p.p.m. to Black Corinth clusters resulted in berries as large as those produced after both girdling and application of growth-regulator.

4. Only the parts of clusters of Black Corinth grapes treated with growth-regulator usually developed large berries and thickened pedicels. The compound, or stimulus induced by it, apparently failed to move from the treated area to any appreciable degree.

DIVISION OF VITICULTURE
UNIVERSITY OF CALIFORNIA
DAVIS, CALIFORNIA

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RESPONSE OF THE COTTON PLANT TO LATE AND LOCALIZED APPLICATIONS OF MALEIC HYDRAZIDE[1]

DAVID R. ERGLE AND WAYNE J. McILRATH[2]

Introduction

In cotton, much as in other plants, the characteristic effect of maleic hydrazide (MH) is inhibition of growth. In earlier studies (**2, 3, 11, 14**) major emphasis was placed on determining the growth responses to various concentrations of MH, 0.1%–0.8%, when applied to the entire aerial portion of the plant during the period of early vegetative development.

This paper deals primarily with general fruiting responses induced by MH when it was applied to the apical leaves and buds of cotton plants during a part of the period of early flowering. The results presented include effects on (*a*) fruiting and vegetative characters, (*b*) yield and properties of fiber, and (*c*) seed viability.

Material and methods

Stoneville 2B cotton was planted October 10, 1950 in forty-five 3-gallon glazed jars containing manured Houston Black Clay and grown one plant to a jar in the greenhouse. When the first flower opened, December 1, 1950, the jars were separated into three groups of fifteen each. The first application of MH was made to plants in two of the groups; the third, serving as a control, was left untreated. The top four or five leaves on the main stem plus the apical bud of each plant in one group were sprayed once weekly for 4 successive weeks with approximately 3 ml. of a 0.2% aqueous solution of MH derived from the diethanolamine salt. Similarly, each plant in the other treatment group received an equal volume of a 0.4% solution. The solutions, containing also 2 ml. of Grasselli spreader per liter, were applied in the mornings with a small hand-operated atomizer in a manner aimed at minimizing runoff to other plant parts. The final applications were made on December 22, 1950.

During the experiment all newly formed flowers were counted and tagged daily. Calculations of boll-shedding percentages were based upon the total number of flowers produced and the corresponding number of bolls retained. Individual plant measurements and counts of other fruiting and vegetative characters were made on February 17, 1951, a few days before the late period of flowering terminated.

Three harvests, representing bolls set the first, second, and third to fifth weeks of flowering, were made of the fiber produced by bolls of early-flower origin. Fiber produced by bolls of late-flower origin was harvested at two dates, representing the seventh to eighth and ninth through eleventh weeks of flowering. Fiber analyses were made by the Cotton Testing Laboratory, Production and Marketing Administration, College Station, Texas.

[1] Approved as Technical Paper no. 1549 of the Texas Agricultural Experiment Station.

[2] Respectively, Chemist, U.S.D.A. and Texas Agricultural Experiment Station, College Station, Texas, and Assistant Professor of Plant Physiology, formerly Department of Plant Physiology and Pathology, College Station, Texas, now Department of Botany, University of Chicago, Chicago, Illinois.

Replicate seed samples from four of the above harvests were germinated in flats of moist sand to determine percentage germination and to check the resulting seedlings for evidence of visible malformations.

Statistical analyses were made by the Statistics Laboratory of the Texas Agricultural Experiment Station.

creased two- and threefold by treatment with 0.2% and 0.4% MH, respectively, and the increases compared with untreated plants were statistically highly significant (table 1).

Both inhibitory and stimulatory responses to MH were found in other fruiting and vegetative characters. Thus, MH at both concentrations limited (*a*) number of fruiting branches, (*b*) plant height, (*c*) number of main-stem nodes, and (*d*) mean length of vegetative branches (table 1). Stimulatory effects were evidenced in lengthening of the fruiting branches and in the increased number of vegetative branches per plant. Most of these changes were of high statistical significance (table 1). Also MH apparently stimulated the formation of vegetative laterals originating on fruiting branches. In the untreated series one plant in the fifteen had one such vegetative lateral, whereas in the 0.2% and 0.4% MH series nine and ten, respectively, of the fifteen treated plants produced one or more of these laterals. Occasionally as many as three such laterals were found at successive nodes on a single fruiting branch of a treated plant.

TABLE 1

EFFECT OF MALEIC HYDRAZIDE (MH) ON REPRODUCTIVE AND VEGETATIVE CHARACTERS OF COTTON: ALL VALUES AS MEANS PER PLANT

Plant characters	Untreated	0.2% MH	0.4% MH
No. of flowers:			
Occurring early	19.27	18.20	16.87
Occurring late	5.73	12.13**	18.93**
No. of fruiting branches	14.13	11.53**	9.53**
No. of vegetative branches	1.47	3.53**	3.93**
Length of fruiting branches (cm.)	15.71	21.48**	23.74**
Length of vegetative branches (cm.)	23.91	14.12**	18.59
Height of main stem (cm.)	113.87	97.67**	83.80**
No. of main-stem nodes	24.73	21.33	17.93**

** Statistically significant, compared with untreated, by odds of 99 to 1.

Results

EFFECT ON FRUITING AND VEGETATIVE CHARACTERS.—The 0.2% applications stopped growth of the main stem without killing the apical bud, whereas those of the higher concentration resulted in death of the bud. Nevertheless, the responses in each case followed similar growth patterns. Early flowering in both treated and untreated plants had ceased by January 4, 1951, and during that time neither concentration of MH had affected significantly the total number of flowers produced per plant. The indicated trend toward a limitation in number of flowers as an effect of MH was not of statistical significance (table 1). Characteristically, flowering started again about January 13, 1951, and continued actively, especially in the treated plants, for a period of 5 weeks. In this latter period the total number of flowers per plant was in-

EFFECT ON BOLL SHEDDING AND YIELD OF SEED COTTON.—Although neither con-

centration of MH had a significant effect on the number of flowers produced during early flowering, both brought about higher boll-shedding percentages than were found for the untreated plants (table 2). The rate of shedding in both the treated and the untreated series increased as the date of boll set advanced, reaching a maximum during the period at 3–5 weeks in which 92%–93% of the bolls in the treated and 78% in the untreated series were shed. At the end of the initial 5-week period 62, 70, and 71% of all the bolls produced, respectively, by the untreated, 0.2% MH-, and 0.4% MH-treated plants, had shed.

The following 5-week period, late flowering, was not subdivided into weeks with respect to boll shed; consequently, weekly shedding rates for that period were not obtained. The net effect was very much the same as that found during early flowering (table 2); 41%–51% of all the bolls produced by the treated plants were shed as compared with 24% of those produced by the untreated ones. The pe-

TABLE 2

INFLUENCE OF MALEIC HYDRAZIDE (MH) ON BOLL SHEDDING AND YIELD OF COTTON
RESULTS BASED ON FIFTEEN PLANTS PER GROUP

Flowering by weeks	Untreated				0.2% MH				0.4% MH			
	No. of flowers produced	No. of bolls set	% shed	Yield of seed cotton (gm.)	No. of flowers produced	No. of bolls set	% shed	Yield of seed cotton (gm.)	No. of flowers produced	No. of bolls set	% shed	Yield of seed cotton (gm.)
	Early flowering											
1st	41	41	0.0	230	48	45	6.2	259	55	52	5.5	246
2d	89	33	62.9	176	82	27	67.1	111	87	16	81.6	36
3–5th	156	34	78.2	149	143	11	92.3	37	122	8	93.4	29
Total, or %	286	108	62.2	555	273	83	69.6	407	264	76	71.2	311
	Late flowering											
7th	1	.....	.....	.....	14	.....	.....	.....	35	.....	.....	.....
8th	1	.....	.....	.....	13	.....	.....	.....	37	.....	.....	.....
9th	12	.....	.....	.....	23	.....	.....	.....	69	.....	.....	.....
10th	23	.....	.....	.....	56	.....	.....	.....	77	.....	.....	.....
11th	43	.....	.....	.....	62	.....	.....	.....	58	.....	.....	.....
Total, or %	80	61	23.8	291	168	99	41.4	400	276	131	51.3	525
	Summary											
Total, or %	366	169	53.8	846	441	182	58.7**	807	540	207	61.7**	836

** Statistically significant, compared with untreated, by odds of 99 to 1.

riod of late flowering in the 0.4% MH-treated plants did, however, differ from that of early flowering in that the total number of flowers produced and the total number of bolls retained increased three and one-half times and twofold, respectively, as a result of the treatment. Similar, but proportionately smaller, increases were induced by the lower concentration of MH.

The yield of seed cotton from bolls set in the early-flowering period was reduced 27% and 44% by 0.2% and 0.4% MH, respectively, compared with that from bolls of the untreated series. Conversely, in the late period of flowering the earlier yield deficits were approximately counterbalanced by increases of from 37% (0.2% MH) to 80% (0.4% MH). Thus, as shown in the summary section of table 2, there was no significant effect of treatment on the combined yields from the two fruiting periods.

EFFECT ON BOLL AND FIBER PROPERTIES.—In the period of early flowering the effect of MH was to reduce the weight of seed cotton per boll, seed index, and lint index (table 3). There were relatively large reductions in both lint and seed weights. MH treatment likewise reduced the weight of seed cotton per boll produced during late flowering, but in this instance most of the decrease was due to the reduction in lint.

As regards fiber properties, treatment with both concentrations of MH tended to result in increases in the length and fineness of fibers from both flowering periods (table 3), but the increases proved to be statistically nonsignificant at the 0.05 level. Likewise, neither concentration of MH had a significant influence on fiber tensile strength.

EFFECT ON SEED VIABILITY AND RESULTING SEEDLING DEVELOPMENT.—Results from germination studies on seeds

TABLE 3

INFLUENCE OF MALEIC HYDRAZIDE (MH) ON BOLL AND FIBER PROPERTIES

Treatment	Weight of seed cotton per boll (gm.)	Seed index (weight of 100 seeds [gm.])	Lint index (weight of lint on 100 seeds [gm.])	Fiber properties				
				Length			Fiber weight per inch (μgm.)	Tensile strength (1000 lb/sq in)
				Upper-half mean (in.)	Mean (in.)	Uniformity ratio		
	Cotton harvested from bolls set during early flowering (values are means of three harvests)							
Untreated	5.14	12.2	6.35	1.14	0.89	78	4.1	81
0.2% MH	4.90	10.6	5.73	1.15	0.90	78	3.6	83
0.4% MH	4.09	8.3	4.86	1.20	0.93	78	3.5	82
	Cotton harvested from bolls set during late flowering (values are means of two harvests)							
Untreated	4.77	12.5	6.67	1.11	0.88	80	4.2	80
0.2% MH	4.04	12.2	6.54	1.12	0.90	81	4.1	79
0.4% MH	4.01	12.2	5.90	1.14	0.91	79	3.9	80

produced by the treated and untreated plants are summarized in table 4. Seeds from bolls of early flowering, at least for the first 2 weeks in that period, were definitely injured by the MH applications, whereas those from bolls of late flowering were not. The percentage germination of seed from the first week of fruiting was lowered from 94.8 (untreated) to 67.6 by 0.4% MH. In addition, a small but significant percentage of the resulting seedlings were malformed. The malformations were limited to the cotyledons and consisted of (*a*) fused petioles, (*b*) fused laminae, or (*c*) both (fig. 1). For the same week the lower concentration of MH was without effect on seed viability, but a small percentage of the resulting seedlings also exhibited the same type of malformations.

At the end of the second week of flowering the treated plants had received two of the four scheduled applications of MH, and the corresponding effects on seed from this period were far more prevalent than those manifested by seed formed earlier. The 0.2% and 0.4% MH treatments reduced the percentage germination from 98 (untreated) to 78 and 14, respectively, and the corresponding percentages of seedlings exhibiting fused cotyledons were 9 and 51 (table 4).

TABLE 4

EFFECT OF MALEIC HYDRAZIDE (MH) ON SEED VIABILITY AND RESULTING SEEDLING DEVELOPMENT

Period of boll set (weeks after treatments started)	Untreated		0.2% MH		0.4% MH	
	% germination	% of resulting seedlings with fused cotyledons	% germination	% of resulting seedlings with fused cotyledons	% germination	% of resulting seedlings with fused cotyledons
	Early flowering					
1st	94.8	0.0	94.4	2.2**	67.6**	2.4**
2d	98.4	0.0	77.6**	8.8**	14.0**	51.3**
	Late flowering					
7th and 8th	98.4	0.0	97.6	0.0	96.8	0.0
9th, 10th, and 11th	98.0	0.0	98.0	0.0	96.8	0.0

** Statistically significant, compared with untreated, by odds of 99 to 1.

Discussion

The results of this study show that MH did not affect significantly the fruitfulness of the cotton plant, as measured by total yield, but that it affected somewhat its usual fruiting behavior. Normally the major part of the total yield is derived from bolls that are set during early flowering on the lower fruiting branches, with the top crop of bolls from late flowering contributing little cotton. As shown (table 2), the plants treated with MH produced most of their cotton from the late crop of bolls at the top of the plant, most of the earlier ones having been shed. This change in the region of heavy fruitset was due largely to a differential effect

of MH on early and late flowering. Thus, early-flower production by the treated plants was essentially the same as that of the untreated ones, plus a greater percentage of shedding, while late-flower production, compared with the untreated, was sufficiently increased on treated plants to more than offset the effects of heavier shedding in both periods.

The number and proportion of vegetative and fruiting branches developed by the untreated plants would appear to be within the usual range of cotton (1). Thus the data in table 1 show that the untreated plants carried approximately one vegetative and fourteen fruiting branches per plant. The effect of MH application, however, was to alter both the number and the proportion of the two types of branches by (*a*) inhibiting elongation of the main stem and consequently reducing the number of fruiting branches and (*b*) by stimulating the formation of additional vegetative branches. The ratio of vegetative to fruiting branches was, respectively, 1:9.6 (untreated plants), 1:3.3 (0.2% MH), and 1:2.4 (0.4% MH). The conclusion is not supported, however, that growth in the treated plants was shifted more toward vegetative than fruiting activity. If there was a change, it appears to have been toward increased fruiting activity, for these fruiting branches (on treated plants), though fewer in number, were longer (fig. 2) and actually produced in the aggregate more flowers and set more but smaller bolls. Moreover, the vegetative branches, though more numerous, were shorter than those carried by the untreated plants.

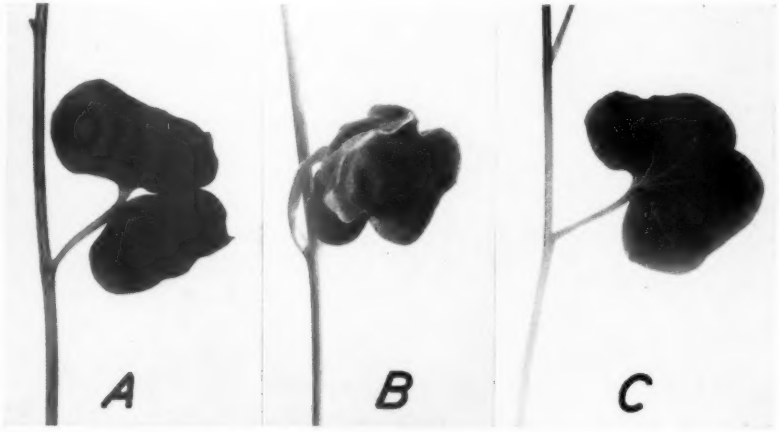

FIG. 1.—Malformed seedlings from seed of MH-treated plants showing cotyledons with: *A*, fused petioles; *B*, fused laminae; and *C*, fused petioles and laminae.

Alteration of the branching behavior of the cotton plant by other factors, such as planting rates, nitrogen supply, and temperature, has been noted by EATON (**4, 7**). He also found (**5**) that the use of the sodium salt of 4-chlorophenoxyacetic acid as a weekly spray resulted in fewer bolls per plant and increased the number of vegetative branches and the weight of stems and leaves. SINGH and GREULACH (**15**), studying the effects of alpha-naphthaleneacetic acid and alpha-naphthaleneacetamide on the development of

Stoneville 2B cotton plants, observed that both vegetative and reproductive development were inhibited.

MH has been shown by others (**9, 10, 13, 14, 16**) to be a growth inhibitor of flower buds as well as of vegetative buds. The results here reported on the flowering of cotton are different, but it should be remembered that both the method and the time of applying MH differed from that usually employed in the study of other plants. It appeared likely that if the MH had been applied generally to the cotton plants a few weeks prior to the first flowering, the flowering response would not have differed significantly from the responses observed in other plants (**11**). As it was, there was no evidence to indicate that sufficient MH was translocated from the leaves to inhibit the development of floral buds formed prior or subsequent to the applications. Movement of MH or its stimulus to the young bolls of early flowering is suggested in the fact that a much higher percentage of those on the treated plants were shed, compared with the untreated plants. Additional evidence of its transport into the developing bolls is shown by the reduction in percentage germination of seeds therefrom.

FIG. 2.—Defoliated treated (0.2% and 0.4% MH) and untreated (C) plants, photographed 80 days after start of treatments, showing effect of MH on length of fruiting and vegetative (tagged) branches. Fruiting bodies shown include floral buds, flowers, young bolls, and locules from which cotton has been harvested. Vacant fruiting nodes resulted from shedding.

Earlier the writers (**12**) showed that 0.01–0.04 mg. of 2,4-dichlorophenoxyacetic acid (2,4-D), applied to the cotton plant at anthesis, injured the developing seed embryos over a 5-week period subse-

quent to the time of application. In addition, larger amounts of 2,4-D (1.0 mg.) reduced germinability of subsequently formed seeds by 65% (**8**). The results of the current investigation, while not indicating a comparable degree of biological activity and persistence, do nevertheless show similarities in the gross effects of the two growth-regulators on developing cotton seeds. As was pointed out in table 4, germination of seed from the MH-treated plants was reduced by as much as 86%, compared with that of seed from untreated plants, and, in addition, half of the resulting seedlings were malformed. However, this effect of MH, in contrast to that of 2,4-D, appeared to be of short duration, as it was not apparent in the seeds originating during the second 5-week period of flowering.

It is well known that the cotton plant in the course of its usual development sheds most of its bolls. The cause of this shedding remains unknown. Data presented elsewhere (**6**) largely eliminate the possibility that boll shedding can be attributed to carbohydrate or nitrogen relations. Although in this experiment treatment with MH caused significant increases in the percentage of bolls shed, the mechanism involved and its relation to the normal shedding of untreated plants remain undefined.

Summary

1. Maleic hydrazide (MH) was applied weekly to the apical buds and leaves of cotton plants during the first 4 weeks of flowering and the effects on (*a*) vegetative and reproductive characters, (*b*) yield and fiber properties, and (*c*) seed viability were studied.

2. MH reduced (*a*) growth in height, (*b*) number of nodes on main stem, (*c*) length of vegetative branches, and (*d*) number of fruiting branches. Its use increased (*a*) the number of vegetative branches, (*b*) length of the fruiting branches, and (*c*) the number of flowers during late flowering. The number of flowers formed in the early period of flowering was not affected. Most of these changes were statistically significant compared with the untreated plants.

3. During the period of early flowering the treated plants set fewer and smaller bolls and consequently yielded less seed cotton than the untreated ones. In the late period of flowering the treated plants set *more* but smaller bolls, and the yield was greater than that of the untreated plants. However, there was no significant effect of MH on either the combined yields from the two fruiting periods or the properties of the fiber.

4. MH injured the seeds that were formed during early (MH applied) but not late (no MH applied) fruiting activity. Injury consisted of reduced germination, as much as 86%, and malformed seedlings.

BUREAU OF PLANT INDUSTRY, SOILS AND AGRICULTURAL ENGINEERING
AND
TEXAS AGRICULTURAL EXPERIMENT STATION
COLLEGE STATION, TEXAS

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RESPONSES OF ORANGE AND GRAPEFRUIT TREES TO MALEIC HYDRAZIDE[1]

LOUIS C. ERICKSON, B. L. BRANNAMAN, HENRY Z. HIELD, AND MILTON D. MILLER

Introduction

Maleic hydrazide (MH) acts as a plant growth-inhibitor when used at certain concentrations and as a herbicide when used at higher concentrations. It has been found (1), for example, that the growth of sugar-beet seedlings is inhibited by a 0.1% spray of MH and that the plants are killed by a 1.0% spray. The exact concentration required for any given effect depends upon the stage of growth of the plant and upon the species.

In testing the response of citrus trees to MH, it was desired to learn whether fruit size or quality might be influenced by the growth-inhibiting properties of this chemical—whether, for instance, an increase in fruit size might be brought about indirectly by delaying the bloom. White (3) delayed the bloom of black raspberries 24–38 days and delayed fruit maturity 16–23 days by an application of MH at the leafing-out stage, before flowers were present. No increase in size of raspberries was noted, but Kuznets and Jennings (2) have shown late bloom in Valencia orange trees to be correlated with large fruit size.

On the other hand, an application of MH to the growing fruit might inhibit development and thereby result in smaller fruit. Larger fruit size of Valencia oranges would be desirable in most districts, but restriction of fruit size of Washington Navel oranges would sometimes be of value when sizes generally are far above average.

Material and methods

A test for delaying bloom was made on Valencia orange and grapefruit trees in an orchard at the Citrus Experiment

[1] Paper no. 731, University of California Citrus Experiment Station, Riverside, California.

Station at Riverside, California. The trees were sprayed on February 28, 1950, prior to the emergence of new growth, with 100, 500, or 1,000 p.p.m. MH.[2] Two trees of each species were sprayed with each concentration, and three trees of each were left as unsprayed checks.

Fruit-drop counts were made for trees of both species, and leaf-drop counts were made for trees of Valencia orange on March 10, March 21, and April 21, 1950. For analysis of fruit quality, to determine whether the composition of the fruit of the current crops had been altered in any way by the sprays, samples of fifty fruits from each Valencia orange tree and of twenty-five fruits from each grapefruit tree were picked on April 19; another sample of twenty fruits was picked from each Valencia orange tree on June 16, 1950. Analyses of fruit quality were also made on the crop of oranges which set after the spray treatments. This last test was made on February 8, 1951, just before the land was cleared of the trees. At this time all fruits on the experimental orange trees were picked, counted, and measured to determine the effects of MH on size and yield. Fifty uniform fruits per tree were used for fruit-quality analysis.

Tests for limiting fruit size were made on Washington Navel oranges in orchards at Riverside, Corona, and Orland, California. At Riverside (in the Bresson orchard) the experimental trees were arranged in a Latin square. Three trees were unsprayed; three were sprayed with 500 p.p.m. and three with 1,000 p.p.m. MH on May 2, 1950, at about full bloom. Fruit was harvested on January 23, 1951. The diameter of each fruit was then measured, and twenty fruits per tree were analyzed for fruit quality.

At Corona (in the Jameson orchard) nine trees in a single row were used for the experiment. In rotation, the trees were unsprayed, sprayed with 100 p.p.m., or sprayed with 500 p.p.m. MH on June 30, 1950, when the average diameter of the larger fruits was 31 mm. At harvest, on February 20, 1951, the diameter of each fruit was measured, and samples of twenty fruits of uniform size per tree were analyzed for fruit quality.

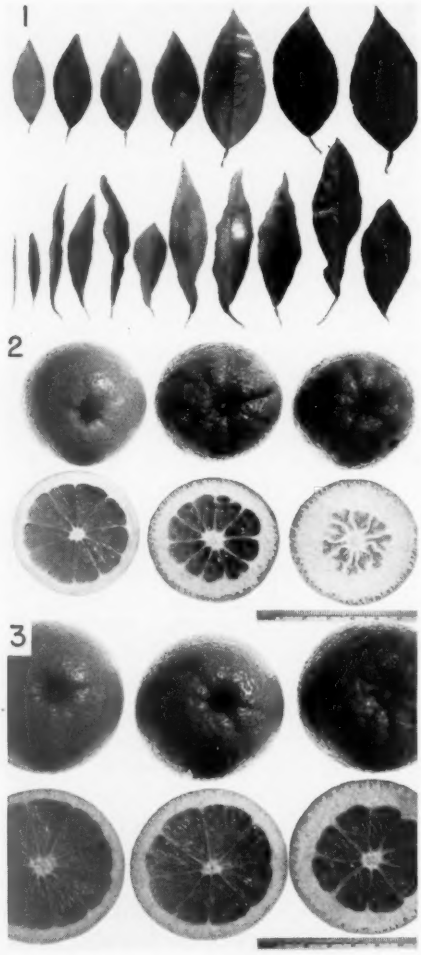
At Orland, experimental trees in two orchards (the Abbott and the Mills orchards) were sprayed with 250, 500, or 1,000 p.p.m. MH or were unsprayed. In each orchard a different tree was sprayed with each concentration of MH on each of the dates given: Abbott orchard, May 11, June 1, and July 3, 1950; Mills orchard, May 10, June 1, July 1, and August 3, 1950. Prior to harvest, ten of the largest fruits on each of four sides of each tree (north, east, south, and west), or forty fruits per tree, were measured for size. A count of the total number of fruits per tree was made at harvest, which occurred on December 13, 1950, in the Mills orchard, and on January 5, 1951, in the Abbott orchard.

Results

WINTER SPRAY TO DELAY BLOOM.—New growth on Valencia orange and grapefruit trees was not visibly delayed by application of a spray containing 100 p.p.m. MH on February 28. Spray containing 500 p.p.m. MH delayed growth by about 1 month, however, on both species, although twigs grew out from time to time during this period, and a few flowers developed on them. Trees sprayed with 1,000 p.p.m. MH produced very few new leaves during the spring and early summer. A few flowers appeared in leaf axils and on the occasional twigs of new growth of these trees during and after the extended flowering period of the control trees. Bloom was sparse on all the trees, whether sprayed or not.

[2] Provided by the United States Rubber Company as a 30% diethanolamine formulation.

As late as July the trees sprayed with 1,000 p.p.m. MH showed scarcely any new growth and were still bearing the dark-green leaves normally present on the trees during the winter. In contrast, the trees sprayed with 500 p.p.m. MH or less had produced a flush of new growth which was still of a relatively light-green color.



FIGS. 1–3.—Fig. 1, Valencia orange leaves: *top row*, normal leaves from unsprayed trees; *bottom row*, narrow, asymmetrical leaves from trees sprayed with 1,000 p.p.m. maleic hydrazide. Figs. 2, 3, mature Washington Navel oranges. Fig. 2, small oranges (size 344): *left*, from unsprayed tree; *center*, from tree sprayed with 500 p.p.m. MH; and *right*, from tree sprayed with 1,000 p.p.m. MH. Fig. 3, medium oranges (size 200) from trees similarly treated. (Note suppressed development of juice vesicles and increased rind thickening in fruit from sprayed trees.)

Formative effects of MH on leaves of both Valencia orange and grapefruit trees were observed in most of the leaves produced in the first new growth after the trees were sprayed with 1,000 p.p.m. of this material (fig. 1). Fewer affected leaves were found on trees sprayed with 500 p.p.m. MH, and none was found on those sprayed with 100 p.p.m.

The characteristic effect of MH on the leaves was to make the blade narrow and asymmetrical. In extreme cases the leaves were linear. Valencia orange leaves showing such modifications were about half again as thick as leaves from control trees. Microscopic examination indicated that increased leaf thickness was due to increased cell size rather than to increased cell numbers. Epidermal, palisade, and spongy parenchyma cells were all proportionately enlarged.

Valencia orange and grapefruit trees in which the spring flush was nearly completely inhibited by MH did not have so much drop of older leaves as did control trees or those sprayed with the lower concentrations (table 1).

A direct effect on abscission might have been postulated from an examination of the leaf-drop counts alone, without considering the association of leaf drop with new growth. Although citrus trees tend to lose a few old leaves from time to time throughout the year, the loss is usually greater following a flush of new growth. Thus, when MH inhibited new growth, it probably indirectly reduced the drop of old leaves normally associated with new growth.

Fruit-drop counts of the current crops of both Valencia orange and grapefruit

trees showed no marked differences which could be attributed to MH at any concentration used.

The total soluble solids in the juice of Valencia oranges showed a significant positive correlation with concentration of spray 49 days after spraying. Other features of fruit quality of the current crops on Valencia orange and grapefruit trees bic acid of the juice may have been reduced to some extent as a result of the MH spray, perhaps because of delayed fruit development.

BLOOM OR POST-BLOOM SPRAYS TO REDUCE FRUIT SIZE.—On Washington Navel orange trees in the Bresson orchard at Riverside, MH spray applied at about full bloom inhibited the summer flush of

TABLE 1

EFFECT OF DIFFERENT CONCENTRATIONS OF MALEIC HYDRAZIDE ON LEAF AND FRUIT DROP OF VALENCIA ORANGE AND GRAPEFRUIT TREES SPRAYED FEBRUARY 28, 1950, TO DELAY SPRING GROWTH*

MALEIC HYDRAZIDE SPRAY (P.P.M.)	LEAF DROP PER UNIT AREA				FRUIT DROP PER TREE			
	Feb. 28 to Mar. 10	Mar. 10 to Mar. 21	Mar. 21 to Apr. 21	Total	Feb. 28 to Mar. 10	Mar. 10 to Mar. 21	Mar. 21 to Apr. 21	Total
	Valencia orange trees							
None (control)	3.3	1.3	38.0	42.6	2.0	2.0	6.7	10.7
100	6.0	6.5	14.5	27.0	2.5	1.5	10.0	14.0
500	3.0	3.5	24.0	30.5	2.0	3.5	4.5	10.0
1,000	2.5	3.0	9.5	15.0	2.5	3.5	8.5	14.5
	Grapefruit trees							
None (control)	2.2	5.2	120.6	128.0	1.4	2.2	5.4	9.0
100	2.7	5.3	158.6	166.6	1.3	3.0	7.7	12.0
500	4.7	6.0	111.4	122.1	1.0	8.3	7.3	16.6
1,000	3.0	5.0	106.3	114.3	2.0	1.8	8.3	12.1

* Citrus Experiment Station orchard, Riverside, California.

at the time of spraying were not subsequently affected, so far as was determined (table 2).

Even though the number of fruits in the succeeding crop of Valencia oranges was much the least on trees sprayed with 1,000 p.p.m. MH, the difference was not significant. There was an inverse correlation between mean size of fruit and concentration of spray, however (table 2). Other characteristics of the next crop were not influenced significantly, although the total soluble solids and ascor-

growth. Fall growth occurred at the normal time, but some of the leaves were modified in shape like those of the sprayed Valencia orange trees.

At maturity, severe modifications of fruit were found in the smallest fruits (fig. 2). Those from trees sprayed with 1,000 p.p.m. MH showed a very much thickened peel (albedo) and almost complete absence of developed juice vesicles; those from trees sprayed with 500 p.p.m. MH showed a condition intermediate between that of the foregoing fruit and nor-

mal. Slightly larger fruits (size 200) showed relatively less rind thickening as a result of the MH sprays (fig. 3).

Large fruit (size 176) sampled for quality showed moderate rind thickening as a result of both the spray concentrations used (table 3). The percentage of juice was decreased and of rind was increased as a result of spraying. The percentage of total acid in the juice was decreased by the spray, while the ratio of soluble solids to acid was increased. The pH value of the juice was positively correlated with concentration of spray.

The juice of fruits sprayed with 500 p.p.m. MH was slightly insipid and off-flavor, and that of fruits sprayed with 1,000 p.p.m. MH was markedly so.

TABLE 2

EFFECT OF DIFFERENT CONCENTRATIONS OF MALEIC HYDRAZIDE ON YIELD, SIZE, AND QUALITY OF VALENCIA ORANGES AND ON QUALITY OF GRAPEFRUIT, FROM TREES SPRAYED FEBRUARY 28, 1950, TO DELAY SPRING GROWTH*

MALEIC HYDRAZIDE SPRAY (P.P.M.)	FRUIT QUALITY						MEAN FRUIT YIELD		MEAN DIAMETER OF FRUIT (MM.)
	Juice (%)	Acid (%)	Total soluble solids (%)	Ratio, soluble solids to acids	pH	Ascorbic acid (mg/100 ml)	Field boxes per tree	Fruits per tree	
	Valencia oranges (current crop, 49 days after spraying)								
None (control)	47.77	1.38	11.44†	8.46	3.27	.....	.....	.....	.....
100	48.71	1.46	11.47†	7.86	3.22	.....	.....	.....	.....
500	47.07	1.41	11.80†	8.40	3.26	.....	.....	.....	.....
1,000	50.25	1.40	11.97†	8.61	3.25	.....	.....	.....	.....
	Valencia oranges (current crop, 108 days after spraying)								
None (control)	51.74	1.09	12.03	11.03	3.49	63.7	.....	.....	.....
100	51.65	1.11	11.74	10.58	3.50	63.4	.....	.....	.....
500	49.17	1.09	11.94	10.95	3.49	62.9	.....	.....	.....
1,000	51.97	1.09	12.03	11.03	3.49	59.7	.....	.....	.....
	Valencia oranges (succeeding crop, 345 days after spraying)								
None (control)	40.50	2.61	16.2	6.28	2.82	93.8	1.37	303	58.7†
100	43.82	2.60	15.8	6.13	2.88	96.6	1.35	273	58.6†
500	45.35	2.70	15.8	5.85	2.88	91.9	1.35	317	56.5†
1,000	41.87	2.30	15.4	6.77	2.87	87.2	0.75	227	52.5†
	Grapefruit (current crop, 49 days after spraying)								
None (control)	43.83	1.66	9.61	5.79	2.99	.....	.....	.....	.....
100	43.26	1.68	9.79	5.83	2.99	.....	.....	.....	.....
500	43.06	1.65	9.59	5.81	3.00	.....	.....	.....	.....
1,000	43.04	1.66	9.54	5.75	2.99	.....	.....	.....	.....

* Citrus Experiment Station orchard, Riverside, California.
† Correlation coefficient significant at 5%.

Although fruit-set on trees sprayed with 500 and 1,000 p.p.m. MH appeared to be less than on unsprayed trees when observations were made during the summer, counts of fruit at harvest time (January 23, 1951) showed that the mean number of fruits per tree was not less, but more, on sprayed trees than on unsprayed trees (table 4). The mean diameter of fruit from sprayed trees was less, however, than that of fruit from unsprayed trees (table 4 and fig. 4), and the correlation coefficient was highly significant.

Fruits from the Jameson orchard at Corona were not so much modified in structure by the 500-p.p.m. MH spray as those from the Bresson orchard at Riverside, probably because the former orchard was sprayed after the fruits were more fully differentiated. Fruit sampled for quality in the Jameson orchard showed only about one-third as much rind thickening as fruit from the Bresson orchard (table 3), and, the increase in rind thickness being slight, the percentage of juice in these fruits was not influenced greatly by the sprays. Nevertheless, the decrease in percentage of juice in fruit of the 500-p.p.m. treatment was consistent enough to show a highly significant statistical difference. A significantly lower ascorbic acid content was found in fruit of the 100-p.p.m. treatment than in fruit of the other treatments, but the validity of this difference is questionable, since fruit of the 500-

TABLE 3

EFFECT OF DIFFERENT CONCENTRATIONS OF MALEIC HYDRAZIDE SPRAY ON QUALITY OF WASHINGTON NAVEL ORANGES

Maleic hydrazide spray (p.p.m.)	Fruit diameter (mm.)	Rind thickness (mm.)	Juice (%)	Rag (%)	Rind (%)	Soluble solids (%)	Total acid, as citric (%)	Ratio, soluble solids to acid	Ascorbic acid (mg/100 ml)	pH	Reducing sugars (mg/ml)	Nonreducing sugars, as glucose (mg/ml)
	Bresson orchard at Riverside (trees sprayed May 2, 1950; fruit harvested January 23, 1951)											
None (control)	73.9	6.4	47.6	9.0	43.4	11.08	1.15	9.61	54.85	3.47	41.2	39.3
500	74.0	8.0	40.0	12.4	47.9	11.28	1.02	11.07	55.26	3.53	44.6	38.5
1,000	74.4	8.4	37.6	12.3	50.1	11.31	0.95	11.92	55.49	3.62	45.9	35.0
L.S.D.												
5%	.....	1.14	——*	.....	2.59	.....	0.14	1.91	.....	——*†	.....	.....
1%	.....	2.63	——*	.....	5.98	.....	0.31	4.40	.....	.....	.....	.....
	Jameson orchard at Corona (trees sprayed June 30, 1950; fruit harvested February 20, 1951)											
None (control)	69.9	6.2	48.87	13.17	37.96	16.13	1.25	12.94	68.52	3.29	66.33	83.31
100	73.0	6.4	49.24	11.70	39.06	15.80	1.22	12.95	64.42	3.31	63.57	77.55
500	71.7	6.7	46.63	14.18	39.19	16.12	1.37	11.79	67.47	3.20	65.73	76.17
L.S.D.												
5%	.....	.....	0.52	.....	.....	.....	.....	.....	3.22	.....	.....	.....
1%	.....	.....	0.86	.....	.....	.....	.....	.....	5.34	.....	.....	.....

* Means did not differ significantly in an analysis of variance, but the factor showed a significant correlation with concentration of spray.

† Statistical treatment of hydrogen ion.

p.p.m. treatment differed only slightly in this respect from fruit of the unsprayed treatment.

Although the greater average number of fruits per sprayed tree in the Jameson orchard was not statistically significant, it appeared to support the trend also shown in the Bresson orchard (table 4).

The Abbott and Mills orchards at Orland also showed a larger number of fruits on sprayed trees than on unsprayed trees. Although not all the fruits on these trees were measured, it was evident that sprayed fruits tended to be smaller than unsprayed fruits selected in a comparable manner (table 4). The quality of fruit from these orchards was not determined.

TABLE 4

EFFECT OF MALEIC HYDRAZIDE SPRAY ON MEAN YIELD AND SIZE OF WASHINGTON NAVEL ORANGES*†

Orchard	Mean fruit yield†				Mean diameter of fruit (mm.)	
	Field boxes per tree		Fruits per tree			
	Unsprayed	Sprayed	Unsprayed	Sprayed	Unsprayed	Sprayed
Bresson (Riverside)	2.3	2.8	309	350	74.4‡	69.5‡
Jameson (Corona)	2.6	2.9	504	618	63.7‡	60.9‡
Abbott (Orland)	.....	.....	614	649	79.4§	76.4§
Mills (Orland)	.....	.....	500	600	76.8§	75.8§

* Orchards at Riverside, Corona, and Orland, California.
† Values for sprayed trees represent all spray concentrations and times of spraying.
‡ Mean of all fruits.
§ Mean of forty large fruits per tree.

Discussion

Maleic hydrazide was effective in delaying the bloom of Valencia orange trees, but, contrary to expectations based on correlation of large fruit size with late bloom (**2**), the mean fruit size of the following crop was reduced rather than increased. Probably, climatological factors responsible for late bloom tend to be associated with other factors that have a bearing on fruit growth. The present experiment was not sufficiently extensive, however, to warrant a final conclusion.

Reduction in mean fruit size owing to bloom or post-bloom spray was to be expected from the inhibiting effects previously reported by many investigators working with MH. An increase in the number of fruits per tree at harvest was not anticipated, however. WHITE (**3**) reported an early drop of fruit from apple trees sprayed in the pink-bud stage. In the present experiments an increased number of oranges was obviously not due to an increased fruit setting, because in most instances the sprays were applied after bloom.

For a period of several weeks after bloom, citrus trees undergo a period of natural fruit thinning. During hot days of early summer this drop is intensified and is known as "June drop," although it is not confined to the month suggested by the name. The thinning frequently extends through July and into August but is usually much less severe as the fruit de-

velops. An increased yield of fruit per tree was apparently effected by maleic hydrazide by slightly depressing the natural thinning processes.

Further investigation will be necessary to find out whether the increased numbers of fruits obtained from sprayed trees can be obtained annually and whether such increases may be of economic importance.

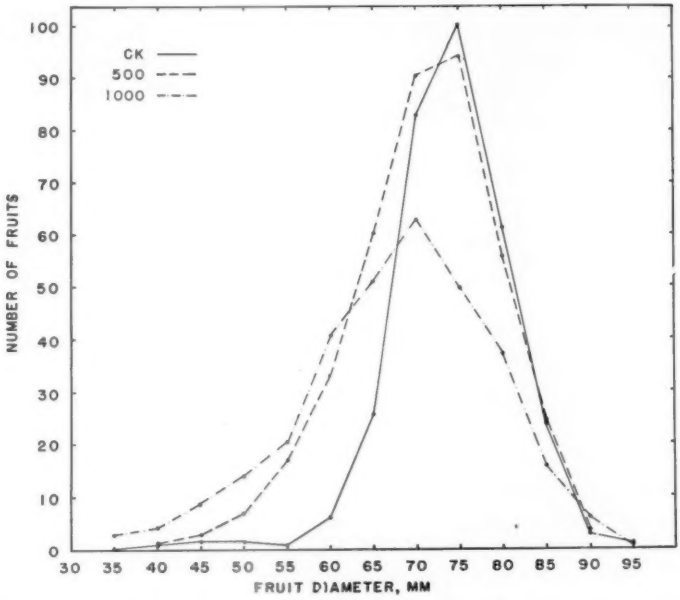


FIG. 4.—Mean size distribution of Washington Navel oranges from trees (three each) unsprayed (control-CK) or sprayed at bloom (May 2, 1950) with 500 p.p.m. or 1,000 p.p.m. MH. Fruit harvested January 23, 1951.

Summary

1. Maleic hydrazide (MH) spray delayed spring growth of Valencia orange and grapefruit trees when applied during late winter at concentrations of 1,000 p.p.m. or 500 p.p.m. but not at 100 p.p.m.

2. Fruit of Valencia orange trees sprayed in late winter showed a correlation between the concentration of MH in the spray and the total soluble solids in the juice. Other factors of fruit quality were not apparently altered. No effects were noted in the quality of fruit from sprayed grapefruit trees. Fruit of the succeeding crop of Valencia oranges had a smaller mean diameter.

3. Fruit of Washington Navel orange trees sprayed with MH at bloom or later showed a reduction in mean fruit size, but there was more fruit per tree on sprayed than on unsprayed trees. Severe modifications of fruit in the form of thickened peel and absence of juice vesicles were found in small fruit from

trees sprayed at bloom. The quality of the fruit from trees sprayed at bloom was altered, and off-flavor was detected. When trees were sprayed several weeks after bloom, no effects on quality of fruit juice were noted.

UNIVERSITY OF CALIFORNIA
CITRUS EXPERIMENT STATION
RIVERSIDE, CALIFORNIA

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FACTORS IN THE DETERMINATION OF THE INTRACELLULAR LOCALIZATION OF ENZYMES[1]

C. E. HAGEN[2] AND VIRON V. JONES[3]

Introduction

Attempts to determine the intracellular distribution of enzymes between the cytoplasm and particulates, or to identify a cellular component that is the principal carrier of an enzymatic activity, have met with difficulties which are associated with the particular techniques employed in the fractionation of cell components. Factors which influence the results are temperature, ionic strength, osmotic pressure, and pH value of the suspending medium, as well as mechanical aspects involved in liberation of the cell contents. The assumption is open to question that the constituents comprising a cellular component will act as a single entity and hence will not exhibit individual responses to these factors.

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[2] United States Department of Agriculture, Agricultural Research Administration, Bureau of Plant Industry, Soils, and Agricultural Engineering, Division of Soil Management and Irrigation Agriculture, Beltsville, Maryland.

[3] Present address: Henry Ford Hospital, Detroit, Michigan.

As an incidental result of another problem being studied in this laboratory, the effect of pH value upon the distribution of some constituents between chloroplasts and solution was very evident. This effect is of some importance with respect to the validity of results obtained by the fractionation technique.

Material and methods

The fractionation of cellular components followed closely the procedure of GRANICK (**4**). Essentially the method consisted of blending 10 gm. of leaves for 20–30 seconds in 100 ml. of a 0.5 *M* sucrose solution, separating the cell debris by pressing the homogenate through four layers of cheesecloth, and isolating the liberated chloroplasts by differential centrifugation. A temperature of approximately 5° C. was maintained for all steps in the procedure. GALSTON (**3**) has shown this technique to be applicable to parallel-veined leaves such as those of oats. Preliminary trials to isolate chloroplasts from spinach, tobacco, millet, tomato, oats, and wheat indicated that such a

procedure yielded morphologically intact plastids from wheat, as shown by microscopic examination. Therefore, Thatcher wheat, 21 days old, was chosen as the source of leaves.

The plants were grown on an organic soil, Michigan muck, which had been fertilized with the major and minor nutrients. The characteristics of the controlled environment were a light intensity of 1,200 foot-candles from a source of alternate white and daylight 30-watt fluorescent light tubes, a temperature maintained at 25° C., and the lights controlled to provide 16 hours of light and 8 hours of darkness.

the leaf suspensions, after blending, ranged in pH from 3.0 to 7.0. The chloroplastic fraction was separated from the cell sap-cytoplasm fraction by differential centrifugation, and the catalase activity of each fraction was determined.

The maximum activity of catalase in cellular fractions from Thatcher wheat is in the pH range of 5.5–7.0 (table 1). The varying distribution of catalase between cell fractions might be due to variation in sorption of the enzyme by the chloroplastic material. This possibility has been tested by preparing a "soluble" catalase extract at pH 6.8, then redistributing the total activity on introduced plastids by

TABLE 1

CATALASE ACTIVITY IN MILLILITERS OF OXYGEN EVOLVED PER GRAM OF FRESH WEIGHT OF THATCHER WHEAT LEAVES

Cell fraction	Apparent pH of blended leaf suspension						
	3.3	4.2	5.0	5.2	5.6	6.0	6.8
Chloroplastic material	6.1	22.6	53.9	55.9	30.2	18.2	21.2
Cell sap-cytoplasm	20.9	8.8	0.0	2.0	216.4	159.3	229.1
Total	27.0	31.4	53.9	57.9	246.6	177.5	240.3

The activity of catalase was determined by addition of aliquots of the material to H_2O_2 in an effective concentration of 0.035 *N*, at pH 6.8 and at 5° C. After a reaction time of 10 minutes the enzyme was inactivated with 2 ml. of 2 *N* H_2SO_4 and the residual H_2O_2 titrated with 0.05 *N* $KMnO_4$. The catalase activity was calculated from the amount of substrate utilized and reported as milliliters of oxygen evolved per gram of fresh weight of leaf.

Results

The effect of pH of the blending medium on the distribution of catalase was tested by liberating the chloroplasts in 0.5 *M* sucrose solutions of pH such that

adjusting the extract to pH 5.0. Apparently the catalase precipitated on the chloroplastic material, which indicates an isoelectric point near that found for beef-liver catalase, 5.58 (**11**) and 5.7 (**12**).

These findings, however, do not preclude the possibility of an initial release of catalase from the particulate, at a pH other than that of its isoelectric point. The precipitation of cytoplasmic proteins in this pH range makes the problem most difficult to study, although an indirect approach to the problem strongly suggests such a possibility.

In the course of isolating the chloroplasts, according to the procedure of GRANICK (**4**) (a pH of 6.5–6.8 for the blended leaf suspensions in 0.5 *M* su-

crose), the supernatant was generally colored green. The absorption spectrum of the supernatant gave absorption maxima at 435 mμ and 675 mμ, which correspond to those of an aqueous extract of the chlorophyll-protein from spinach (**10**). Presumably, this green aqueous extract is the product designated by STOLL and WIEDEMANN as "chloroplastin." Other workers have reported the isoelectric point of such chlorophyll-protein complexes to vary from pH 3.7 for the algae *Chlorella* and *Oscillatoria* (**5**) to pH 5.0 for white clover (**6**).

TABLE 2

PERCENTAGE TRANSMISSION OF AQUEOUS CHLOROPHYLL-PROTEIN EXTRACTS*

WAVE LENGTH IN Mμ	APPARENT pH OF 0.5 M SUCROSE SUSPENSION MEDIUM					
	2.0	3.0	4.0	5.0	6.0	7.0
435...	67.0	98.5	99.0	71.0	35.0	31.8
675...	81.6	99.5	100.0	79.4	47.0	43.0

* 0.1-ml. chloroplast aliquot; 25 ml. total suspension volume; temperature 0° C.

Since both chlorophyll and the iron-porphyrin prosthetic group of catalase are associated with specific protein carriers, the effect of pH on the retention of the chlorophyll-protein complex in the chloroplasts was tested. Aliquots of isolated chloroplasts were resuspended in a series of 0.5 *M* sucrose solutions at pH ranging from 2.0 to 7.0. A period of 60 minutes was required for the green color of the "solution" to approach a maximum. The chloroplastic material was separated from solution by centrifugation, and the transmission of the supernatant (table 2) was determined on a Beckman spectrophotometer.

SISAKYAN and KOBYAKOVA (**8**) have suggested that most of the enzymes in the plastids are adsorbed but are released to solution upon autolysis, with the more strongly bound exhibiting the least activity prior to the autolysis. In a study of peroxidase and polyphenoloxidase activity of the sugar beet, SISAKYAN and KUVAEVA (**9**) demonstrated the effect of osmotic pressure of the suspension medium on "desorption" of enzymes from the leucoplasts. In short, a minimum "desorption" occurred at a 0.8 *M* mannitol concentration, with progression to a maximum at 0.1 *M*. The total effect was reflected in an apparent rise of enzyme activity in vitro with increasing osmotic pressure.

To test the possibility of similar effects with catalase a leaf homogenate was prepared in order that the "desorption" of catalase might be favored. No pH adjustment of the sucrose medium was made prior to blending. After blending, the suspension was immediately adjusted to pH 6.9 and allowed to stand at 5° C. in an ice bath. Aliquots were withdrawn periodically, and the catalase activity was determined (table 3).

TABLE 3

CATALASE ACTIVITY IN MILLILITERS OF OXYGEN EVOLVED PER GRAM OF FRESH WEIGHT OF THATCHER WHEAT LEAVES

MINUTES OF PRETREATMENT AT APPARENT pH OF 6.9						
15	45	75	135	195	255	315
278.0	235.1	239.4	322.8	404.3	408.5	400.5

Discussion

The validity of histochemical methods to indicate intracellular enzyme distribution appears to be in doubt owing to artifacts arising from diffusion and adsorption of both substrates and enzymes (**7**). Apparently, pH value is one of the

factors which may give rise to such artifacts (**7**).

Likewise, in the cell-fractionation technique for determining the intracellular location of enzymes, the virtual impossibility of isolating particulates which are free from contamination of cytoplasmic constituents suggests a severe limitation on such studies. A pH which is near neutral has been shown to be favorable for reducing the precipitation of cytoplasmic fractions with plant particulates separated by centrifugation (**4**). CLAUDE (**1**) has shown that the large granules and microsomes of mammalian liver agglutinate below a pH of 7.0. If the acidity is too great or if the particulates are allowed to remain in a near-neutral or alkaline medium, the separation of the various elements is no longer possible by differential centrifugation. The same difficulty was encountered by DOUNCE (**2**) in a study of enzymes associated with isolated cell nuclei of rat liver. In the present study a comparable agglutination of suspensions of Thatcher wheat chloroplasts was apparent at pH 4.0–5.0. This parallels the optimum pH for precipitation of isolated oat chloroplasts from suspensions (**3**). Thus, a necessary condition for fractionation of cell components by differential centrifugation appears to be a near-neutral pH of the separating media.

Because of this general limitation of technique the assumption that all the constituents comprising a particulate will act as a single entity and will not exhibit individual responses to the pH value of the extracting medium is open to question.

The pH of the blending medium in which Thatcher wheat chloroplasts were liberated was shown to influence the apparent distribution of catalase between chloroplasts and the cell sap-cytoplasm fractions. At an acid pH the evident precipitation of cytoplasmic fractions which cannot be separated from the particulates by differential centrifugation has led to a tentative conclusion that the constituents are initially in the cytoplasm and are sorbed on the particulate under these conditions. An alternate interpretation of such data is that release or "desorption" of the component initially present in the particulate is due to a pH effect on the system. Because of the inherent nature of the systems produced by cell fractionation, the answer is not obtainable by direct experimentation with the present techniques.

Evidence as to the effect of pH on the release of a component of a particulate to the solution is suggested by the study of the chlorophyll-protein. There appears to be no doubt that the chlorophyll-protein complex is associated with the grana of chloroplasts, in vivo. Such a condition has been found to exist in the cell-fractionation technique only at a pH which is apparently in the vicinity of the isoelectric point of the complex. If prejudice did not rightfully exist in associating the chlorophyll-protein with the chloroplasts, entirely erroneous conclusions as to its location would be drawn from techniques which employed a near-neutral pH to avoid contamination of precipitated proteins.

The evidence of a "desorption" of catalase in a leaf suspension at a near-neutral pH is open to some question. However, since recent work (**8, 9**) has shown other enzymes to be "desorbed" from plastids into the soluble state, the release of catalase to solution seems quite plausible.

Although these data are critical of the interpretation of much of the experimental work on the intracellular localization of enzymes by the cell-fractionation tech-

nique, a solution to this problem is not offered here.

Summary

1. Distribution of catalase among cell fractions of wheat leaves was dependent on the pH of the sucrose medium in which the cell components were liberated by blending. The catalase activity was all associated with the chloroplastic material at a pH of 5.0 and was mostly in the soluble fraction at a pH of 3.3 or 5.6.

2. Chloroplastin, the chlorophyll-protein of the plastids, was likewise dependent in its distribution on the pH of the suspension, essentially complete retention by the plastids being found at pH 3.0 to 4.0.

3. The catalase activity of the suspension at a pH of 6.9 increased with time, which suggests a "desorption" of the enzyme from particulates.

4. These results emphasize that the constituents of a cellular component will respond individually to variations of a factor such as pH in cell-fractionation procedures.

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EFFECTS OF BETA-NAPHTHOXYACETIC ACID ON FRUIT SIZE IN THE MARSHALL STRAWBERRY[1]

QUENTIN B. ZIELINSKI AND RALPH GARREN, JR.

Introduction

During the past decade numerous papers have appeared dealing with responses obtained with growth-regulating substances applied to horticultural plants. These papers show that such phenomena as date of bloom, fruit-set, fruit size, abscission, rate of coloring, and rate of maturity may be influenced by such chemicals. MARTH and MEADER (**1**) and WALDO and HANSEN (**3**) obtained marked increases in the size of blackberry

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fruits by spraying the blossoms with growth-regulators, but in these tests the treatments sometimes resulted in epinasty, flower injury, and malformation of fruits. Beta-naphthoxyacetic acid applied as a late spray is now used to some extent as a means of inducing increased size in Hawaiian pineapples. The experiments reported in the present paper were initiated to determine whether applications of beta-naphthoxyacetic acid made at some time after bloom would be effective in inducing increase in size of strawberry fruits and, at the same time, would avoid the deleterious effects that may accompany early application of such compounds.

Material and methods

The experiments were conducted in 1951 during the strawberry flowering and maturation season. The plants were 2 years old and were growing on the Experiment Station farm at Corvallis, Oregon. These were vigorous plants of the Marshall variety, which had produced an abundant crop of early blossoms. Sections of rows 35 feet in length were selected for each of the treatments, and ten plants of uniform size were designated for close observation in each section. One individual fruit on each plant was selected and tagged for detailed growth measurements. In selecting these fruits, an attempt was made to choose specimens that were similar as to size, position on the inflorescence, number of fruits on the inflorescence, and exposure to light. Since the experiments were largely of an exploratory nature, no attempt was made to replicate the various treatments.

The sprays in the first series of tests were applied on May 24 and consisted of various concentrations (10, 25, 50, and 100 p.p.m.) of an aqueous solution of beta-naphthoxyacetic acid. The solutions were prepared by dissolving a weighed amount of the acid crystals in a small amount of 95% alcohol. To this was added tap water containing 0. 05% of Tween 20 (manufactured by Atlas Powder Co.). Application was made with a small compressed-air hand sprayer, using a graduating Vermorel nozzle. The entire plant was covered with the spray, but particular care was taken to insure complete coverage of the inflorescences.

The berries tagged for measurement in this series were approximately half-grown when the sprays were applied, and thus were probably treated at from 15 to 18 days after pollination. The initial measurements were made 3 days after spraying, and subsequent measurements were made at 4-day intervals thereafter until the fruit attained full maturity. The measurements, which included both the polar and the equatorial axes of the fruit, were made with a Vernier caliper graduated in centimeters. The growth increment of both diameters for each period of measurement was calculated by finding the means of the sums of the polar and equatorial axes of the ten fruits and subtracting therefrom the value obtained in the initial measurement.

On June 12 a second series of treatments was initiated, the sprays being applied to fruits which had developed from a late crop of blossoms on plants not previously sprayed. The individual fruits tagged for measurement were somewhat smaller than were those of the first series. The spray concentrations were also similar except that the 10-p.p.m. concentration was omitted.

Observations were made from time to time as to dates of maturity, color of fruit, dessert quality, and comparative freedom from epinasty, imperfect fruits, and other abnormal symptoms. Twice during the progress of each series, ma-

ture fruits were selected for the determination of total solids by the refractometer method.

Results

MAY 24 SERIES.—All tagged fruits which had been sprayed were larger at the ripening stage than were the tagged fruits from the controls, except for those which received the 10-p.p.m. concentration (fig. 1). Expressed as percentage of increment over the controls, the treated fruits gave the following values: 10 p.p.m., no increase; 25 p.p.m., 16.5% increase; 50 p.p.m., 32.7% increase; and 100 p.p.m., 25.9% increase. Apparently the 10-p.p.m. concentration was too low to bring about a response, whereas the 100-p.p.m. concentration seems to have induced a depressing effect.

In this series the sprayed fruits reached maturity and attained maximum size at about the same time as did the fruits on the controls. The sprayed fruit showed no inhibition of color or flavor, nor did its total-solids content show a significant difference from that of the controls.

The sprayed plants displayed no symptoms of epinasty or other deleterious effects that could be attributed to the sprays. On the other hand, the foliage of sprayed plants had a more intense green color than that of plants which had not been treated.

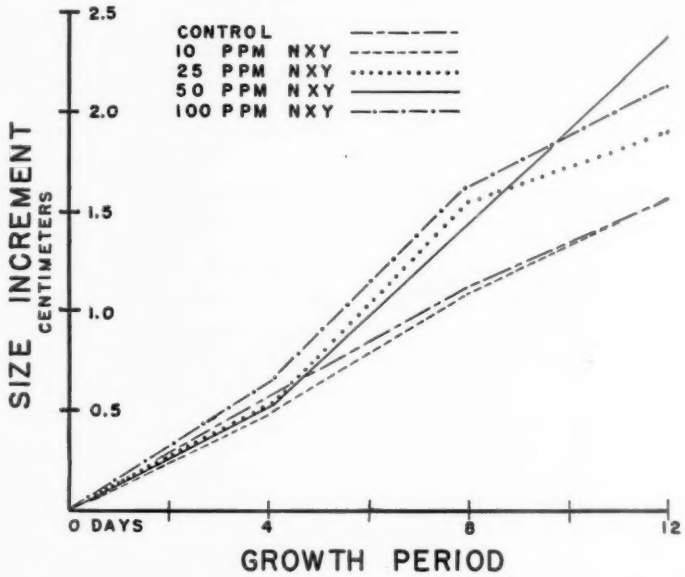

FIG. 1.—Influence of beta-naphthoxyacetic acid sprays on fruit-size increment in Marshall strawberries, May 24 series. Each increment figure represents mean value of polar and equatorial axes of ten fruits minus value of initial measurement. Initial measurements were made 3 days after spraying.

JUNE 12 SERIES.—In the series initiated on June 12, all the sprayed plants produced fruits (fig. 2) that were larger in size than those from plants from the unsprayed plots, although the increments were of lesser magnitude than those recorded for the May 24 series. The size in-

crements over those of the controls for the June 12 series were as follows: 25 p.p.m., 12.1%; 50 p.p.m., 23.5%; and 100 p.p.m., 20.5%.

The differences in size increment between the two series can probably be accounted for by the differences in weather conditions that prevailed during the respective periods. It was cool and moist during the period covered by the May 24 series, whereas the opposite was true in the second series. The mean temperature averaged approximately 10° F. higher during the second series, and, under these conditions, strawberry fruits throughout western Oregon generally failed to attain the size usually expected when weather conditions are more favorable.

In contrast to the observations on the first series, some differences were noted among the various treatments of the June 12 series as to the time during which maximum fruit size was attained. The fruits from the controls and from the 25-p.p.m. treatment reached maximum size about 12 days after spraying, fruits from the 50-p.p.m. treatment in about 8 days, and fruits from the 100-p.p.m. treatment in about 16 days.

Observations on fruit quality made during the second series were practically the same as those of the first. The fruits showed no significant differences as to color and dessert quality among the various treatments or between the treatments and the controls. No objectionable "seediness" developed in any of the lots, and no appreciable differences were recorded in the percentages of total solids. The plants which had been sprayed again showed no symptoms of injury.

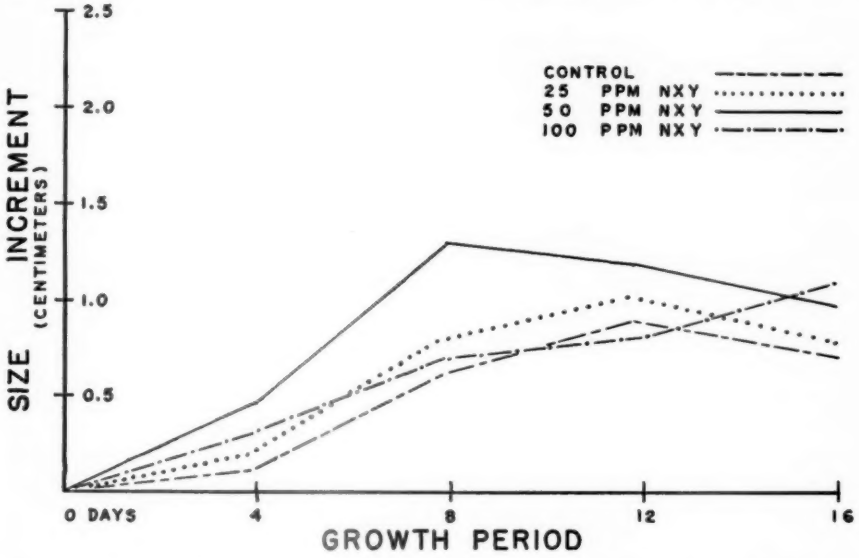

FIG. 2.—Influence of beta-naphthoxyacetic acid sprays on fruit-size increment in Marshall strawberries, June 12 series. Increment figures were computed as in figure 1. Initial measurements, in this series, were made at time of spraying.

Discussion

Among the encouraging aspects of the results are the lack of deleterious effects from the sprays. Both fruit and plants re-

mained free of immediate symptoms of injury even when fairly high concentrations were applied. In supplemental tests to determine tolerance, concentrations as high as 500 p.p.m. failed to result in visible injury to either fruit or plants.

In perennial plants, however, the effects of growth-regulators may sometimes carry over from one year to the next. This makes it imperative that work be done to ascertain the likelihood of cumulative effects in the case of the strawberry. Recommendations for the practical use of beta-naphthoxyacetic acid cannot be made until the possibility of deleterious cumulative effects has been shown to be nonexistent.

Whether through coincidence or bona fide correlation, the data from these experiments tend to substantiate the interpretations of NITSCH (**2**) relative to the influence of auxin or a growth-regulating substitute on the development of strawberry receptacles. The time of application of the sprays in the present experiments corresponds closely to the period when, according to NITSCH, the auxin content of the achenes can be expected to show a sharp decline. If it is true that auxin from the achenes is responsible for the growth of the receptacles, then it is plausible that applications of beta-naphthoxyacetic acid made at some time after bloom may act in lieu of auxin and induce increase in size beyond normal expectancy.

Although these experiments must be regarded as preliminary, the data are sufficiently striking to warrant additional research. Tests should be carried on involving replicated plots and quantitative determinations of yields to substantiate fully the results pertaining to increment of fruit size. It might also be of practical value to explore the possibility of applying the chemical more than once during the blooming and fruiting season. Since there is indication that drought may tend to limit action of the chemical, testing its effect in relation to varying soil moisture gradients might be worth while. The increased intensity of the green foliage color observed in the sprayed plants also merits further study, since it suggests a possible relationship between the action of the chemical and the nutritional functions of the plant. This intensification of green coloration following the application of various growth-regulators is of common occurrence but as yet has not been explained.

Summary

1. The action of beta-naphthoxyacetic acid on the growth of strawberry fruits subsequent to flowering has been studied. Applications of this chemical in the form of sprays applied some time after pollination gave significant increments of fruit size. From a series of tests begun on May 24, the following increments over controls were obtained: 10 p.p.m., no increase; 25 p.p.m., 16.5% increase; 50 p.p.m., 32.7% increase; and 100 p.p.m., 25.9% increase. A second series of tests set up on June 12 produced similar results except that the percentages of increase were somewhat lower.

2. Under the conditions of these experiments no harmful effects from the sprays were noted on either the fruits or the plants. This was true even of sprays applied in fairly high concentrations.

3. Although the results obtained are indicative of possible field and commercial significance, additional work must be done before the use of the chemical can be recommended as a practice in strawberry culture.

OREGON AGRICULTURAL EXPERIMENT STATION
CORVALLIS, OREGON

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SECRETION OF PHOSPHORYLASE BY RED KIDNEY BEAN EMBRYOS[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY **636**

S. M. SIEGEL

Experimentation

In the course of investigations using the isolated embryonic axis of the Red Kidney bean (*Phaseolus vulgaris*), it was observed that around both axis and cotyledons, when cultured on sterile starch–agar, there developed an achromatic zone on application of dilute IKI solution. The zone of degraded starch ranged from two to five times the actual area of contact of tissue and substrate.

Dormant axes placed on the agar produced a distinct zone in about 24 hours at 24° C.; isolated axes incubated in darkness for 2–3 days prior to use (at which time endogenous carbohydrates had almost completely disappeared) produced a larger achromatic zone in 12 hours than dormant individuals had in 24 hours.

These observations were confirmed in several experiments, and in all cases the achromatic zone approximated the outline of the entire embryonic axis or cotyledon.

The phosphorylase activity of carbohydrate-depleted axes was demonstrated by floating 0.5-mm. sections of hypocotyl in 5 ml. of a buffered, aqueous solution of glucose-1-phosphate (3–5 mg/ml). After incubation for 1 hour at 24°–26° C. slices at pH 4.5 and 5.6 showed a definite increase in staining with IKI; after a reaction time of 4 hours sections at the above-mentioned pH values stained intensely with iodine, whereas sections at pH 6.4 showed a much lighter starch test, and those at pH 8.8 were negative. Controls were (*a*) a solution of Cori ester without tissue slices and (*b*) slices in buffer solutions without glucose-1-phosphate.

When examined under the microscope, sections were found to give the starch test in epidermis, cortex, endodermis, and pith; the vascular cylinder was devoid of starch. A particularly intense reaction was observed in the epidermal and endodermal cells. The endogenous starch supply of the embryonic axis differed in distribution from that of starch synthesized in slices in being absent from the epidermis.

When the intact embryonic axis was substituted for slices, similar pH relations were observed. In this case five axes were placed in 3 ml. of Cori ester (5 mg/ml), and the solution was tested for starch after 4 hours' incubation. The

[1] This work was supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

color (reddish brown) was progressively lighter in the following order of pH values: 5.6, 4.5, 6.4, 8.8 (negative).

Carbohydrate-depleted axes were incubated on an agar medium containing 2–5 mg. of glucose-1-phosphate/ml under sterile conditions. The upper portion of each hypocotyl was dipped in petrolatum to seal off the cotyledonary attachment prior to placing the axes on this substrate. After an incubation period of 5 or more hours the iodine test revealed that polysaccharide yielding a red-brown amylose color had been synthesized in the agar.

In all likelihood precautions taken to eliminate loss of phosphorylase through broken cells were needless because axes on agar containing glucose-1-phosphate synthesized less polysaccharide in the region of cotyledonary attachments than in the root and lower portions of hypocotyls.

The secretion of other enzymes by the embryonic axes was investigated. Peroxidase and catalase, both demonstrably present in sections, were absent in agar diffusates. Results of tests for urease activity have not been conclusive but suggest that the enzyme may be released from intact tissue.

Discussion

It is generally known that cells of many organisms are capable of secreting enzymes. GALL (**2**) in 1948 demonstrated that sections of bean-stem tissue were capable of degrading starch when incubated on a starch-agar medium; he interpreted his observations as being the result of release of amylase and/or phosphorylase from the wounded surface of the tissue. BRAKKE and NICKELL (**1**) recently reported the release of alpha-amylase from tissues of a virus-free tumor taken from the roots of *Rumex acetosa*. Their figures indicate release of a starch-degrading enzyme(s) from intact cells; however, their claim that the enzyme in question was an amylase is not based on conclusive experimental evidence, inasmuch as they failed to show that their preparation was unable to synthesize starch or to release inorganic phosphate from Cori ester or other suitable substrates. Reference may also be made to a report dealing with extracellular activity of phosphatase and other enzymes in corn and tomato roots (**3**).

The molecular weight of crystalline peroxidase is one-tenth that of crystalline rabbit phosphorylase *a* (**4**). If a difference of this magnitude obtains in the molecular weights of bean embryo peroxidase and phosphorylase, secretion of the latter must be a regulated function rather than a "leakage."

In view of the evidence presented it may be concluded that secretion of phosphorylase, and perhaps other enzymes, is a common function of the bean embryo. No obvious role is suggested for the phenomenon as it occurs in the tissue of this germinating seed. Possibly enzyme secretion is of significance during the early development of the embryo when movement of carbohydrates and other materials might be facilitated by extracellular enzymes.

Summary

1. The secretion of phosphorylase by intact tissues of the Red Kidney bean embryo was demonstrated. Both degradation of starch and synthesis of amylose from glucose-1-phosphate were observed; several aspects of starch synthesis were examined. Neither peroxidase nor catalase was secreted, but urease may be released by the embryos.

2. It is suggested that the phenomenon of enzyme secretion is of significance in development of embryos.

DEPARTMENT OF BOTANY
UNIVERSITY OF CHICAGO

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SEPARATION OF D-GLUCOSE FROM THE HYDROLYSIS PRODUCTS OF SCENEDESMUS CELLS

JOHN W. E. GLATTFELD

The procedure reported here was used for the separation of D-glucose in crystalline form from the products of the dilute sulfuric-acid hydrolysis of *Scenedesmus* cells. The technique is valuable because (*a*) there is, to the author's knowledge, no report in the literature of the isolation in crystalline form of glucose from the hydrolysis products of *Scenedesmus* cells, and (*b*), if labeled (with C^{14}) cells were used as the starting material, the procedure should yield randomly labeled glucose of high specific activity, as the use of "carrier" glucose would not be necessary for separation of the labeled glucose.

The cells were harvested by centrifugation and dried at 80° C. They were extracted exhaustively, first with 80% ethanol and then with absolute methanol. They were then stored in a vacuum desiccator over sulfuric acid until the weight was constant.

In the hydrolysis experiment 50 gm. of extracted cells were used with 250 cc. of 1 *N* sulfuric acid; the refluxing period was 20 hours. The part that did not go into solution was separated by centrifugation; it weighed 17.3 gm. after drying, so that, by difference, 32.7 gm. of the sample dissolved.

The supernatant liquid was freed of sulfate ion by means of barium hydroxide. After the barium sulfate was removed by centrifugation, the liquid was passed through a column of the monobed resin, Amberlite MB-3. Since the color of the solution was not removed by the Amberlite, the effluent was passed through a decolorizing resin, Permutit-DR, which removed all color. It was then again passed through Amberlite MB-3. It had been previously shown with pure glucose solutions that neither Amberlite MB-3 nor Permutit-DR retains more than 10% of the sugar. The volume of the clear, colorless effluent was 6,600 cc. The solution contained, by analysis, 6.0 gm. of reducing material calculated as glucose. It was distilled at reduced pressure and left 7.43 gm. of clear, but slightly yellow, stiff gum. Since this contained 6.0 gm. of material calculated as glucose, the sugar content was 80.7%.

The gum was then dissolved by heating it with a mixture of 6 cc. of water and 60 cc. of absolute ethanol, and the solution was filtered to remove a small amount of flocculent material. The filtrate was crystal clear but faintly yellow. It was seeded with glucose crystals, and the sides of the flask were scratched with a glass rod. Almost 18 hours elapsed before crystalliza-

tion started along the scratches. It then proceeded rather rapidly. Twenty-seven hours after the time of seeding the first crop of crystals was harvested. It was pressed down on the suction filter but not washed. The mother-liquor and rinsings were distilled to dryness at reduced pressure, the residue was again taken up in a mixture of one part water and ten parts absolute ethanol, and the procedure outlined above was repeated. In this way a total of 1.92 gm. of white crystalline material was obtained in three crops.

The 1.92 gm. of crude material was recrystallized from a mixture of 2 cc. of water and 20 cc. of absolute ethanol and yielded 1.0 gm. of snow-white granular crystals with the following constants:

$[\alpha]_D^{20}$ at equilibrium	+52.7
$[\alpha]_D^{20}$ (accepted value for glucose)	+52.5
M.P. (° C.)	145°–146°
M.P. of an authentic sample of glucose	146°–147°
M.P. of a mixture of the two	145°–146°

I am indebted to Mr. RONALD WATANABE, a member of the laboratory staff, for the preparation of a chromatogram of the recrystallized material; it showed only the presence of glucose with no other contaminating sugars.

These results leave little doubt that the sugar thus separated from the hydrolysis products of *Scenedesmus* was D-glucose.

DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH
ARGONNE NATIONAL LABORATORY
CHICAGO, ILLINOIS

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